

**RAMIFICATIONS OF MICROGLIAL ACTIVATION  
ON OLIGODENDROCYTES IN *IN VITRO* MODELS  
OF MULTIPLE SCLEROSIS**

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**A thesis submitted for the degree of  
Doctor of Philosophy (Ph.D.)**

I, Ioanna Sevastou confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

## Acknowledgements

I would like to sincerely thank my supervisor Dr Jennifer Pocock for giving me the opportunity to work in her lab as well as for her guidance, support, enthusiasm and encouragement throughout this PhD project. I would also like to thank my secondary supervisor Professor Simon Heales for always being there for me.

Many thanks to my colleagues, Dr Vicky Fry, Dr Claudie Hooper, Dr Emma East, Ms Emma Mead, Mr Thomas Piers, Mr Adamantios Mamais, Ms Ruth Faram and Ms Frida Laulund for their help and friendship.

A big thank you to AIMS2CURE for generously funding this project.

Last but not least I would like to thank my mother Aggeliki, my brother Giorgos and my friends, for their immense support, understanding, encouragement and advice throughout these three years.

## Abstract

Oligodendrocytes are the principal target of immune attack in multiple sclerosis (MS), where the immune system attacks the myelin sheath, leading to oligodendrocyte demise, compromised axonal saltatory conduction and eventually neuronal degeneration. Microglia are also implicated in MS pathology and are present in lesions in an activated state. To study the effects of activated microglia on oligodendrocyte precursor cells (OPCs) or mature oligodendrocytes, primary cultures of microglia, oligodendrocytes, or co-cultures of the two cell populations were developed. Microglia activated with lipopolysaccharide (LPS), were toxic to OPCs and mature oligodendrocytes in co-culture. This toxicity was mediated by tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ) released from microglia. Microglial activation with the blood-borne proteins (BBPs) fraction V albumin, fibrinogen and fibrin was also investigated. BBPs enter the brain after blood-brain barrier disruption, a typical event in MS, so their differential effect upon microglial activation and subsequently oligodendrocyte lineage cell maturation survival was studied. BBPs had distinct effects on microglial activation and oligodendrocyte cell toxicity. Fraction V albumin and fibrin were directly toxic to oligodendrocytes while microglial activation protected oligodendrocytes in co-culture. Fibrin also inhibited OPC maturation into myelinating oligodendrocytes. Fibrinogen activated microglia were toxic to OPCs and mature oligodendrocytes in co-culture. Attenuation of microglial induced oligodendrocyte death was attempted by blocking pathways of microglial activation such as the Rho-ROCK pathway and by modulating microglial activation by metabotropic glutamate receptor manipulation. Additionally, ROCK inhibition was able to attenuate LPS or BBP activated microglial expression of inducible nitric oxide synthase (iNOS). Modulation of microglial activation could prevent microglial induced oligodendrocyte toxicity and could lead to strategies to slow disease progression in MS patients by protecting mature oligodendrocytes from microglial induced death, or most importantly by enhancing survival and maturation of OPCs in MS lesions, where their recruitment to remyelinate neuronal axons is vital for disease remission.



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## Abbreviations

<b>°C</b> - degrees Celsius	<b>DCGIV</b> - (2S,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl) glycine
<b>AC</b> - adenylyl cyclase	<b>dH<sub>2</sub>O</b> - distilled water
<b>AIDA</b> - (RS)-1-aminoindan-1,5-dicarboxylic acid	<b>DHA</b> - docosahexaenoic acid
<b>ALS</b> - amyotrophic lateral sclerosis	<b>DHPG</b> - (S)-3,5-dihydroxyphenylglycine
<b>AMPA</b> - $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid	<b>DIV</b> - days <i>in vitro</i>
<b>AMT-HCl</b> - 2-Amino-5,6-dihydro-6-methyl-4H-1,3-thiazine hydrochloride	<b>DMEM</b> - Dulbecco's modified Eagle medium
<b>ANOVA</b> - analysis of variance	<b>DMSO</b> - dimethyl sulfoxide
<b>APICA</b> - (RS)-1-amino-5-phosphonoindan-1-carboxylic acid	<b>DNA</b> - deoxyribonucleic acid
<b>APS</b> - ammonium persulfate	<b>DPT/NO</b> - dipropylenetriamine-NONOate
<b>AraC</b> - cytosine arabinoside	<b>DETA/NO</b> - diethylaminetriamine-NONOate
<b>ATP</b> - adenosine 5'-triphosphate	<b>EAE</b> - experimental autoimmune encephalitis
<b>ATPase</b> - ATP hydrolysing enzyme	<b>EBV</b> - Epstein-Barr virus
<b>AU</b> - arbitrary units	<b>EBSS</b> - Earle's balanced salts solution
<b>BBB</b> - blood-brain barrier	<b>ECL</b> - enhanced chemiluminescence
<b>BBP</b> - blood-borne protein	<b>EDTA</b> - ethylene diamine tetra-acetic acid
<b>BDNF</b> - brain-derived neurotrophic factor	<b>eGFP</b> - enhanced green-fluorescent protein
<b>BM</b> - basic medium	<b>EGTA</b> - ethylene glycol tetraacetic acid
<b>BMP4</b> - bone morphogenetic protein 4	<b>ELISA</b> - Enzyme-linked immunosorbent assay
<b>BSA</b> - bovine serum albumin	<b>ER</b> - endoplasmic reticulum
<b>BSI-B<sub>4</sub></b> - <i>Bandeiraea simplicifolia</i> isolectin B <sub>4</sub>	<b>EU</b> - endotoxin units
<b>cAMP</b> - cyclic adenosine monophosphate	<b>FasL</b> - Fas ligand
<b>CG-4</b> - central glia -4	<b>FBS</b> - foetal bovine serum
<b>CGA</b> - chromogranin A	<b>FCS</b> - foetal calf serum
<b>CGC</b> - cerebellar granule cell	<b>FITC</b> - fluorescein isothiocyanate
<b>CM</b> - conditioned medium	<b>FG</b> - fibrinogen
<b>CNP</b> - 2',3'-cyclic nucleotide 3'-phosphodiesterase	<b>FGF</b> - fibroblast growth factor
<b>CNS</b> - central nervous system	<b>FN</b> - fibrin
<b>CNTF</b> - ciliary neurotrophic factor	<b>FV</b> - fraction V albumin
<b>COX-2</b> - cyclooxygenase-2	<b>g</b> - acceleration due to gravity
<b>CS-PG</b> - chondroitin sulphate proteoglycan	<b>g</b> - gram
<b>CSE</b> - control standard endotoxin	<b>GABA</b> - $\gamma$ -aminobutyric acid
<b>CSF</b> - cerebrospinal fluid	<b>GalC</b> - galactocerebroside
<b>CTR</b> - control	<b>GDNF</b> - glial-derived neurotrophic factor
<b>DAG</b> - diacylglycerol	<b>GFAP</b> - glial fibrillary acidic protein
<b>DAPI</b> - 4',6-diamidino-2-phenylindole	<b>Glu</b> - glutamate

**GTP** - guanosine triphosphate  
**GTPase** - guanosine triphosphate hydrolysing enzyme  
**h** - hour  
**HEPES** - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid  
**HIV** - human immunodeficiency virus  
**HLA-DR** - human leukocyte antigen-DR  
**HRP** - horse radish peroxidase  
**ICAM** - inter-cellular adhesion molecule  
**IFN $\gamma$**  - interferon- $\gamma$   
**IGF** - insulin-like growth factor  
**iGluR** - ionotropic glutamate receptor  
**IL** - interleukin  
**iNOS** - inducible nitric oxide synthase  
**IP<sub>3</sub>** - inositol triphosphate  
**JAM** - junctional adhesion molecule  
**K** - thousand  
**KA** - kainate  
**kDa** - kilodalton  
**L** - litre  
**LAL** - Limulus Amebocyte Lysate  
**L-AP4** - L-(+)-2-amino-4-phosphonobutyric acid  
**LPS** - lipopolysaccharide  
**M** - molar  
**MAdCAM-1** - mucosal addressin cell adhesion molecule-1  
**MAG** - myelin associated glycoprotein  
**MAP** - mitogen-activated protein  
**MAP4** - (S)-2-amino-2-methyl-4-phosphonobutanoic acid  
**MAPK** - mitogen-activated protein kinase  
**MBP** - myelin basic protein  
**MEM** - minimum essential medium  
**MFN** - mitofusin  
**MG** - microglia  
**mg** - milligrams  
**MGCM** - microglial-conditioned medium  
**mGluR** - metabotropic glutamate receptor  
**MHC** - major histocompatibility complex  
**min** - minute  
**ml** - millilitre  
**mM** - millimolar  
**MMP** - matrix metalloproteinase  
**MOG** - myelin oligodendrocyte protein  
**mRNA** - messenger ribonucleic acid  
**MS** - multiple sclerosis  
**MW** - molecular weight  
 **$\mu$ g** - microgram  
 **$\mu$ l** - microliter  
 **$\mu$ M** - micromolar  
**NAAG** - N-acetylaspartylglutamate  
**NADPH** - nicotinamide adenine dinucleotide phosphate  
**NF- $\kappa$ B** - nuclear factor kappa-light-chain-enhancer of activated B cells  
**ng** - nanogram  
**NGS** - normal goat serum  
**nM** - nanomolar  
**NMDA** - N-methyl-D-aspartic acid  
**nNOS** - neuronal nitric oxide synthase  
**NO** - nitric oxide  
**ns** - not statistically significant  
**NSAID** - non-steroidal anti-inflammatory drug  
**NT-3** - neurotrophin-3  
**OL** - oligodendrocyte  
**OPC** - oligodendrocyte precursor cell  
***p*** - probability value  
**PAGE** - polyacrylamide gel electrophoresis  
**PAMP** - pathogen-associated molecular pattern  
**PBS** - phosphate buffered saline  
**PDGF** - platelet-derived growth factor  
**PDGF $\alpha$ R** - platelet-derived growth factor  $\alpha$  receptor  
**PDL** - poly-D-lysine  
**PECAM** - platelet endothelial cell adhesion molecule  
**pg** - picogram  
**pH** - hydrogen ion concentration  
**PI** - propidium iodide  
**PKA** - protein kinase A  
**PKC** - protein kinase C  
**PLC** - phospholipase C  
**PLP** - proteolipid protein  
**PMX** - polymyxin B

**pNA** - p-nitroaniline  
**PSA-NCAM** - polysialylated neural cell adhesion molecule  
**PP-MS** - primary progressive multiple sclerosis  
**PNS** - peripheral nervous system  
**PVDF** - polyvinylidene di-fluoride  
**ROCK** - Rho-associated coiled coil-containing protein kinase  
**ROS** - reactive oxygen species  
**rpm** - revolutions per minute  
**RR-MS** - relapsing-remitting multiple sclerosis  
**RSPPG** - (RS)-4-phosphonophenylglycine  
**RT** - room temperature  
**RTPZ** - phosphatase- $\beta/\zeta$   
**SCM** - serum-containing medium  
**SD** - standard deviation  
**SDS** - sodium dodecyl sulphate  
**SEM** - standard error of the mean  
**SFM** - serum-free medium  
**SIB-1757** - 6-methyl-2-(phenylazo)-3-pyridinol  
**SIN-1** - 3-morpholiniosydnonimine  
**SNAP** - S-nitroso-N-acetylpenicillamine  
**SNP** - sodium nitroprusside  
**SNP** - single nucleotide polymorphism  
**SOD1** - Cu/Zn superoxide dismutase  
**SP-MS** - secondary progressive multiple sclerosis  
**SR101** - sulforhodamine  
**SSeCKS** - src-suppressed C-kinase substrate  
**T3** - triiodothyronine  
**T4** - thyroxine  
**TEMED** - N, N, N', N'-tetramethylethylenediamine  
**Thal** - thalidomide  
**TLR** - Toll-like receptor  
**TNF- $\alpha$**  - tumour necrosis factor  $\alpha$   
**tPA** - tissue plasminogen activator  
**TREM** - triggering receptor expressed on myeloid cells  
**TRITC** - tetramethyl rhodamine isothiocyanate  
**TTBS** - Tween-20 Tris buffer saline  
**U** - unit  
**UV** - ultraviolet  
**VCAM** - vascular cell adhesion molecule  
**VEGF** - vascular endothelial growth factor  
**w/v** - weight/volume

## **1. INTRODUCTION**

## **1.1 Cells of the nervous system in health and disease**

### **1.1.1 Structure and function of the nervous system**

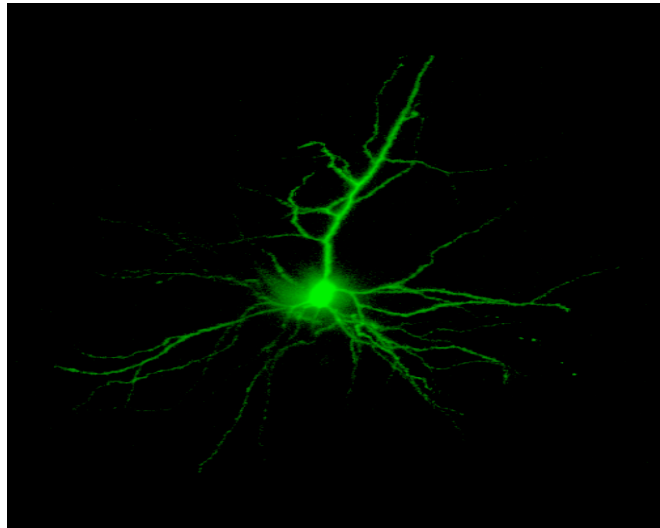
The nervous system is the organ responsible for information processing and communication with the external world. The nervous system's complex functions are achieved by a great number of neuronal cells that form an even greater number of connections between them. It was estimated that the average human brain contains about 85 billion neurones (Lange, 1975; Azevedo et al., 2009) with synapses in the neocortex alone estimated at around 164 trillion (Tang et al., 2001). The neurones are supported, maintained and protected by the glial cells. There are an estimated 85 billion non-neuronal cells in the human brain (Azevedo et al., 2009). The glial types in the brain are microglia, astrocytes, oligodendrocytes and NG2 glia (Nishiyama et al., 2002). Anatomically the mammalian nervous system can be divided into the central and the peripheral nervous system. The central nervous system (CNS) is comprised of the brain and the spinal cord whilst the peripheral nervous system (PNS) consists of motor and sensory nerves that run from the CNS to the muscles and sensory organs of the body.

The brain is an extremely sensitive organ, thus many evolutionary adaptations have securely placed it in the head. The brain is surrounded by the cranial bone, which is strong and resists vibration and impact (McElhaney et al., 1970). In a similar manner, the spinal cord runs through the vertebral column guarded by 33 vertebral bones. Under the cranial bone and vertebral column, the brain and spinal cord are enveloped by three meninges, the dura mater, the arachnoid mater and the pia mater. The subarachnoid space between the arachnoid and the pia matter is filled with cerebrospinal fluid (CSF). CSF provides a neuroprotective function as a hydraulic cushion for the brain and spinal cord. It likely also serves metabolic, nutritional, immunologic, and scavenging functions for the CNS (reviewed by Kapoor et al., 2008). Further to the anatomical protective structures, the CNS is an immunologically privileged site protected from external insults such as infections and toxic molecules by the blood-brain barrier (BBB), a cellular barrier formed by the endothelium of blood vessels entering the brain (see 1.4.1).

Protective structures and strategies have evolutionarily developed to protect the CNS, alas, it is not invulnerable to trauma and disease. The CNS is prone to neurogenetic and metabolic diseases, developmental disorders, neuroinflammatory and neurodegenerative diseases of adult life, cerebrovascular diseases, convulsive disorders, infections and brain tumours. When attempting to unravel the mechanisms of brain function in health and disease and develop rational therapeutic approaches to address CNS disease and trauma, basic and clinical research are two equally important components that contribute to the elucidation of physiological mechanisms and the experimentation on mechanisms that could have therapeutic benefits.

### **1.1.2 Neurones**

Neurones are the cells of the nervous system responsible for receiving, processing, storing and transferring information. Neurones are electrochemically excitable cells that can produce electric currents and communicate with other cells in an excitatory or inhibitory manner by releasing excitatory and inhibitory neurotransmitters (reviewed by Seal & Edwards, 2006). A typical neurone consists of the nucleus containing soma, branched cellular extensions where the majority of input to the neurones occurs, known as dendrites, and the neuronal axon (Figure 1.1.2.1). The neuronal axon is a long and thin formation that can extend significantly in length. For example the sciatic nerve can be over a metre long. The CNS tissue is distinguished into grey matter, which contains neural cell bodies, and white matter, which mostly contains axon tracts.



**Figure 1.1.2.1 Typical neurone**

Confocal microscopy image of a neonatal rat pyramidal neurone stained with Lucifer Yellow, image size 320 x 425 x 120  $\mu\text{m}$ . The neuronal soma, apical dendrite (extending upwards) dendrites and the nerve axon extending downwards and slightly to the left can be viewed. Image adapted from the Wadsworth Centre image gallery, <http://www.wadsworth.org/cores/alm/gallery.htm>.

The unique function of the nerve axon is owed to the presence of voltage gated ion channels,  $\text{Na}^+/\text{K}^+$ -ATPase,  $\text{Ca}^{2+}$ -ATPase and  $\text{Na}^+/\text{Ca}^{2+}$  exchangers on the plasma membrane (reviewed by Waxman & Ritchie, 1993). The axon maintains an internally negative membrane potential of -60 millivolts due to the specific permeability of its membrane to  $\text{K}^+$  which leads to  $\text{K}^+$  leakage out of the neurones. Nerve cells are electrically excitable because depolarisation of the cell membrane beyond threshold value of +40 millivolts elicits action potentials that can be conducted down the axon towards the nerve terminal (Hodgkin & Huxley, 1952). At the nerve terminal, the axon forms specialised structures named synapses, where the action potential causes depolarisation and release of neurotransmitters. The neurotransmitters are released into the synaptic cleft, where succeeding neurones or effector cells can be excited (reviewed by Catterall, 1984).

Neurones are vulnerable to neurodegenerative disease and in some cases specific neuronal types or populations are selectively affected, without conclusive views on why. For example, in amyotrophic lateral sclerosis (ALS), a type of motor neurone disease, motor neurones selectively degenerate and die. Approximately 10% of classical ALS is familial, with approximately 20% of familial ALS caused by dominantly

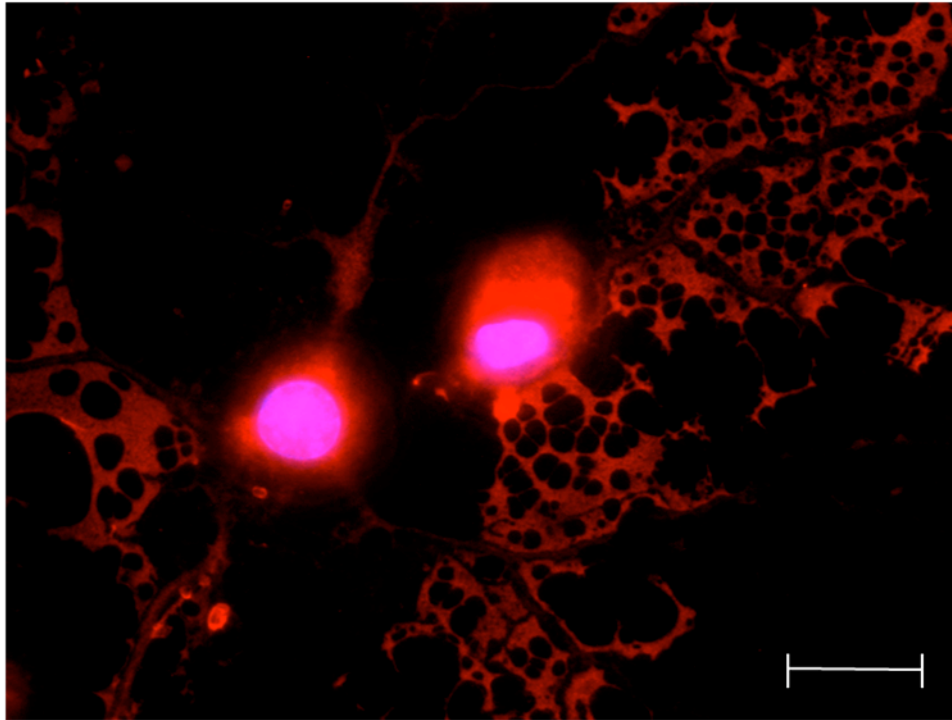


inherited mutations in the protein Cu/Zn superoxide dismutase (SOD1; Rosen et al., 1993). The pathological and clinical similarity between familial and sporadic disease sparked enthusiasm that the animal models based on mutant SOD1 could provide insight into mechanisms of both sporadic and familial disease. However, to date, there is no direct evidence validating this assumption (reviewed by Bruijn et al., 2004). In the case of Parkinson's disease, there is deterioration of the dopaminergic neurones of the substantia nigra, a basal ganglial region that projects to the striatum to control movement, accompanied by a well-described pathophysiology of  $\alpha$ -synuclein neuronal inclusions in specific regions. Still the aetiology of the disease remains elusive (reviewed by Lees et al., 2009). In Alzheimer's disease, there is specific deterioration of neurones in the cerebral cortex and subcortical areas in conjunction with amyloid plaques and neurofibrillary tangles present, again without clear aetiology and with little therapeutic advances (reviewed by Hardy, 2006). There are though neurodegenerative diseases where aetiology is clearer. An example is Charcot-Marie-Tooth neuropathy type 2, an axonal disease, which manifests slow nerve conduction velocity, muscle atrophy and loss of sensation. Mutations in the nuclear encoded mitochondrial GTPase mitofusin (MFN) 2 cause most cases of the disease (Züchner et al., 2004).

Neuronal vulnerability to insults results in chronic dysfunction. Neurodegeneration is in most cases slow and starts developing before disease symptoms occur, making prognosis and early treatment difficult to achieve. Research into neurodegeneration is vast and there is hope that unravelling the molecular mechanisms of neuronal function will help address neurodegeneration management and cure.

### 1.1.3 Oligodendrocytes, the nerve axon myelinating cells

Oligodendrocytes are the glial cells of the CNS dedicated to the insulation of nerve axons. They are terminally differentiated cells that form long processes packed with myelin. Myelin is a highly lipidic compact membrane with a low proportion of protein (15-30%) that wraps spirally around axons to form segments of myelin sheath. The lipidic composition of myelin differs from any other biological membrane and contains cholesterol, phospholipid, galactolipid and plasmalogen at an approximate molar ratio 2:2:1:1 (Norton & Poduslo, 1973). The most abundant myelin proteins are proteolipid protein (PLP; 30–45% of total myelin protein), myelin basic protein (MBP; 2–35% of total myelin protein) and 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase; 4–15% of total myelin protein; reviewed by Jahn et al., 2009). Antibodies raised against myelin proteins can be used to immunochemically label mature oligodendrocytes *in vitro* and *in vivo*. Proteins that constitute less than 1% of total myelin proteins, such as myelin oligodendrocyte protein (MOG) and myelin associated glycoprotein (MAG) are also suitable for staining mature oligodendrocytes (Linnington et al., 1984). The myelin membrane is not as inert as initially considered and has been shown to express receptors for growth factors, hormones and cytokines, such as the oestrogen receptor and caveolin-1, a negative regulator of endothelial nitric oxide synthase (Arvanitis et al., 2004). Oligodendrocytes *in vitro* extend long, branched processes and express myelin proteins (Figure 1.1.3.1).



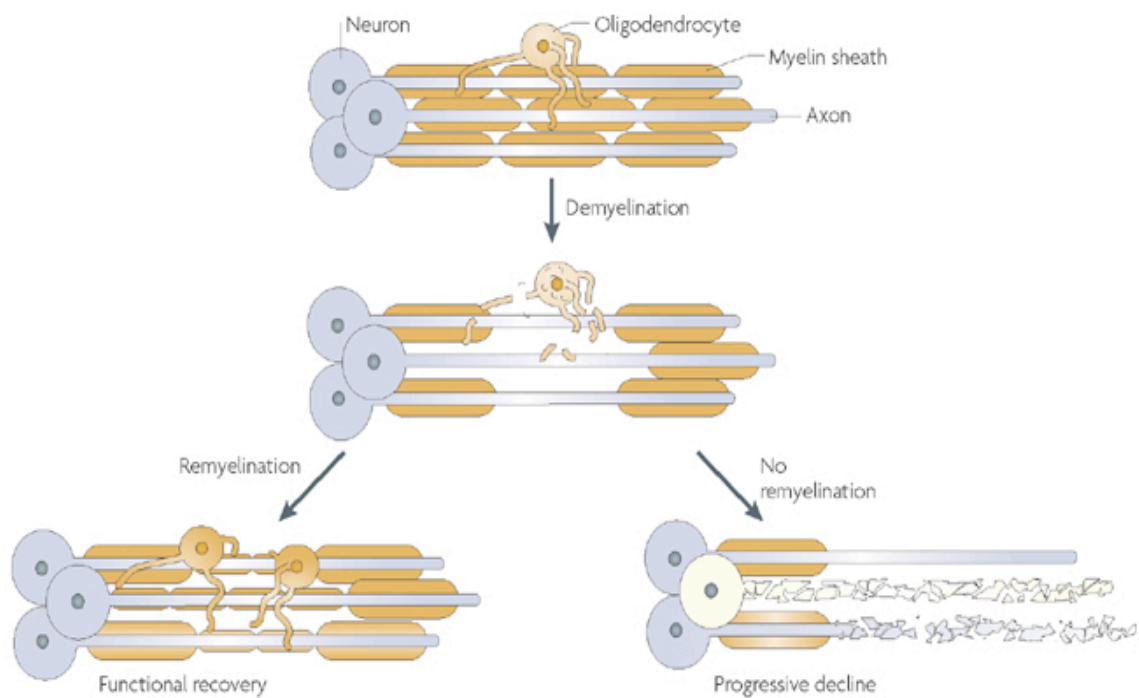
**Figure 1.1.3.1 Oligodendrocytes *in vitro***

Image of 6 day *in vitro* (DIV) cultured oligodendrocytes *in vitro* stained with anti-myelin basic protein antibody (MBP, red) and the nuclear stain 4',6-diamidino-2-phenylindole (DAPI, blue). The long highly branched fine processes typical of oligodendrocytes *in vitro* can be viewed. Scale bar = 20  $\mu\text{m}$ .

Myelin facilitates the efficient conduction of the nerve impulse by insulating the nerve axon. The myelin sheath normally covers all the axon, with periodic interruptions between myelin segments, the nodes of Ranvier (reviewed by Susuki & Rasband, 2008). At the nodes of Ranvier electrical resistance is low and there is a high concentration of  $\text{Na}^+$  channels. Therefore depolarisation is facilitated, the nerve impulse is renewed and it can travel fast through the next myelinated segment. This procedure is known as saltatory conduction and has evolutionarily developed to ensure fast and continuous propagation of the nerve impulse (Schweigreiter et al., 2006). The  $\text{Na}^+$  channels at the nodes of Ranvier cluster during myelination, the procedure that drives oligodendrocytes to make contact with nerve axons and start wrapping them with myelin sheaths. Before myelination, the  $\text{Na}^+$  channels  $\text{Na}(\text{v})1.2$  cluster diffusely along the axon, guided by oligodendrocyte derived signals (Kaplan et

al., 2001). During myelination, oligodendrocyte derived factors seem to be responsible for the expression of Na(v)1.6 channels that cluster at the nodes of Ranvier substituting for the Na(v)1.2 channels that remain scattered on the axons (Boiko et al., 2001). This procedure shows how myelinating oligodendrocytes direct myelination and formation of the myelin sheath. One oligodendrocyte extends multiple processes and is able to myelinate up to 50 axons. At the same time many oligodendrocytes are required to myelinate one axon, each providing a myelin sheath section between two nodes of Ranvier (Figure 1.1.3.2).

The nervous system can be subjected to demyelinating disorders. Demyelination is the pathological process by which the myelin sheath is directly insulted and disturbed or destroyed, exposing the nerve axons and interrupting transmission. This leads to axonal degeneration and neuronal death (reviewed by Franklin & French-Constant, 2008). The most common demyelinating disorder is multiple sclerosis (MS), which will be discussed in Chapters 2 and 3. Genetic abnormalities that affect oligodendrocyte lineage cells, collectively named leukodystrophies also result in demyelination (reviewed by Schiffmann & van der Knaap, 2004). A diagrammatic representation of myelinated and demyelinated axons and their fates following demyelination can be seen in Figure 1.1.3.2.



**Figure 1.1.3.2 Schematic representation of myelination processes in the CNS**

Schematic representation of neurones with extended aligned nerve axons myelinated by oligodendrocytes. One oligodendrocyte can myelinate many axons as seen in the first sketch. Following demyelination oligodendrocytes degenerate leaving axons exposed. These axons will either be remyelinated by oligodendrocyte precursor cells (see 1.1.4) or will degenerate and die. Figure adapted from Franklin & French-Constant, 2008.

#### 1.1.4 Oligodendrocyte precursor cells (OPCs), NG2 glia and remyelination

Oligodendrocyte precursor cells (OPCs) are the cells of the CNS that give rise to oligodendrocytes during development. Platelet derived growth factor AA (PDGF-AA) and fibroblast growth factor -2 (FGF-2) are two growth factors specifically required to specify differentiation of cells of the ventral neural tube into OPCs (Fruttiger et al., 1999; Kessaris et al., 2004). OPCs are small, bipolar, motile cells that can be identified by the A2B5 monoclonal antibody (Raff et al., 1983). Platelet derived growth factor  $\alpha$  receptor (PDGF $\alpha$ R) and the sulphated proteoglycan NG2 are two more specific markers that have been used to identify OPCs *in vitro* (Nishiyama et al., 1996). After OPCs migrate to the region where they will differentiate, their migratory ability is lost, accompanied by expression of the O4 marker, their first step into differentiation (Warrington et al., 1993). At this point the cells are referred to as pre-oligodendrocytes and proliferate further under the combined guidance of PDGF-AA and FGF-2 in a complex state of molecular events, until O4 expression is substituted for O1 expression. By this point the cells have lost their proliferative ability and have made multiple contacts with nerve axons (Back et al., 2002). Cell maturation is completed by the sequential expression of oligodendrocyte and myelin specific components, such as galactocerebroside (GalC) and the myelin proteins MBP, MOG, MAG and PLP, followed by the myelination of neighbouring nerve axons with long myelin-packed processes (Figure 1.1.4.1). Growth factors that facilitate the process of myelination are thyroid hormone thyroxine (Baas et al., 2002), erythropoietin (Sugawa et al., 2002) and neurotrophin-3 (NT-3; Rubio et al., 2004). Intracellularly, activation of Src family Fyn tyrosine kinase (Osterhout et al., 1999), protein kinase C (PKC) and mitogen-activated protein kinase signalling are required for successful myelination (reviewed by Stariha & Kim, 2001).

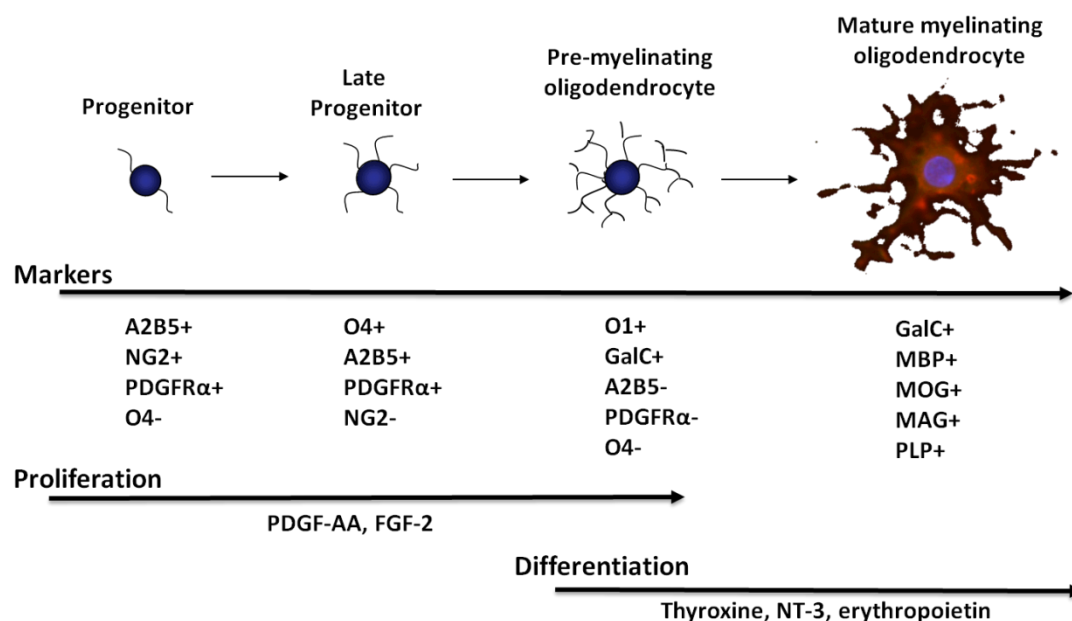
In the adult CNS, a population of cells resembling OPCs, with a slow rate of migration and a long cell cycle, remains and proliferates actively (Wolswijk & Noble, 1989). These cells make up around 3-9% of the glial cell population in the adult CNS and are known as NG2 glia, since they stain with the OPC specific marker NG2 (Dawson et al., 2003). Their function is still under intense research. In the healthy

brain they seem to have a regulatory role. In the rat spinal cord NG2 glia were shown to actively proliferate and differentiate into 1% of mature oligodendrocytes and astrocytes over a 4-week period (Horner et al., 2000). A similar study in a cell culture system derived from human brain temporal lobe cells revealed that 1.3 % of the cells cultured had OPC properties, whilst there were also 3 % O4 positive cells present. *In vitro*, NG2 cells act as O-2A progenitors and differentiate into A2B5 and GFAP (astrocyte marker) positive cells and less often into oligodendrocytes (Scolding et al., 1995). It should be noted here that *in vitro*, in the presence of serum, OPCs, often referred to as O-2A cells, can give rise to both oligodendrocytes and type 2 astrocytes which are cells that express both astrocytic markers and markers of the oligodendrocyte lineage (Raff et al., 1983). A recent study unravelled the molecular profile of human brain white matter NG2 cells and identified that inhibition of the receptor tyrosine phosphatase- $\beta/\zeta$  (RTPZ) signalling pathway induced mature oligodendrocyte production, whilst bone morphogenetic protein 4 (BMP4) signalling resulted in astrocyte production (Sim et al., 2006).

In response to demyelination, NG2 glia acquire a remyelinating role, migrate to the site of injury and commence remyelination (French-Constant & Raff, 1986). Remyelination is the process by which the destroyed myelin sheaths are replaced around demyelinated axons and saltatory conduction is restored (Smith et al., 1979). Tissue is fully regenerated in remyelination, with the only difference to normal tissue being that remyelinating cells construct thinner and smaller myelin sheath segments (Ludwin & Maitland, 1984; Figure 1.1.3.2). Adult brain OPCs (NG2 cells) as well as stem cells originating from the subventricular zone can migrate to the site of injury and differentiate into mature oligodendrocytes (reviewed by Franklin & French-Constant, 2008). The two different steps required for remyelination are migration and proliferation of the precursors, followed by their switch from a migratory-proliferating phenotype to a differentiating one. The factors that regulate these two phenotypes are distinct and inhibitory to each other. PDGF-AA and FGF-2 are crucial for OPC rapid proliferation (Murtie et al., 2005), while FGF-2 also inhibits OPC differentiation (Zhou et al., 2006).

Even though the CNS has an innate regenerative mechanism, remyelination is not always successful and with MS as a striking example, it does indeed fail on many

occasions. As demonstrated by the presence of quiescent OPCs in chronically demyelinated areas of the brain (Wolswijk, 1998), remyelination failure is more likely due to failure in OPC differentiation rather than failure in OPC recruitment. There is thus much research interest into the reasons that render the demyelinating environment inhibitory to remyelination (see 1.3.3).



**Figure 1.1.4.1 Development of the oligodendrocyte lineage cells**

Schematic representation of the major steps in oligodendrocyte precursor cell maturation. Some of the markers that identify the developmental status of the cell are listed. Oligodendrocyte precursor cells proliferate and migrate under the guidance of platelet-derived growth factor AA (PDGF-AA) and fibroblast growth factor 2 (FGF-2). Pre-myelinating oligodendrocytes stop proliferating and enter the myelination process, which is facilitated by thyroxine and the growth factors neurotrophin-3 (NT-3) and erythropoietin, resulting in mature oligodendrocytes with myelin packed processes. Abbreviations: PDGFRα, platelet-derived growth factor receptor α; GalC, galactocerebroside; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; MAG, myelin associated glycoprotein; PLP, proteolipid protein.



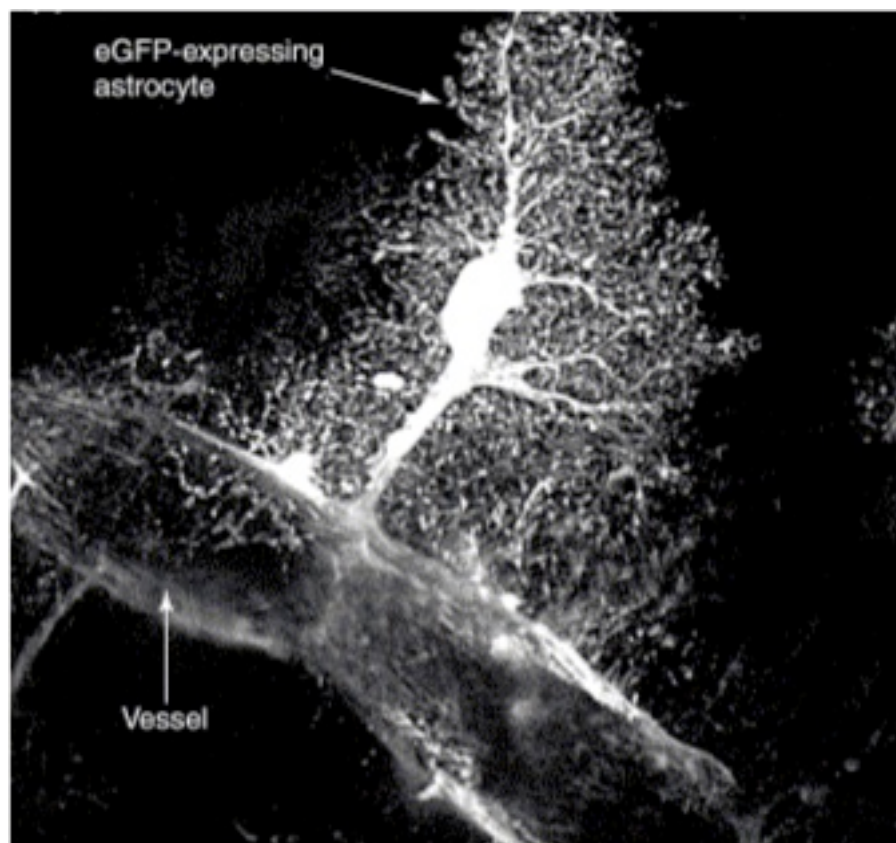
### 1.1.5 Schwann cells

Schwann cells are the myelinating cells of the PNS. In contrast to their CNS counterparts, the oligodendrocytes, they maintain their ability to de-differentiate, re-enter mitosis, proliferate and give rise to new myelinating cells following demyelination and injury. Unlike oligodendrocytes, each Schwann cell only myelinates one axon by folding around it. Schwann cells in the mature PNS can be myelinating or non-myelinating, ensheathing large and small diameter axons respectively (reviewed by Jessen & Mirsky, 2005). During development the immature Schwann cells are not required for the development of the nerve axons but do offer trophic support to developing neurones (Riethmacher et al., 1997).

Schwann cells are targeted in inherited disorders of the PNS such as Charcot-Marie-Tooth (especially type 1) and the Guillain-Barré Syndrome, leading to extensive demyelination of peripheral nerves. The use of Schwann cells for CNS remyelination has been proposed, since they retain their ability to proliferate and remyelinate, and are also not targeted by auto-antibodies against oligodendrocytes, a characteristic event in MS. Schwann cell transplantation to improve remyelination following spinal cord injuries has been attempted with encouraging results (Kohama et al., 2001; Pearse et al., 2004).

### 1.1.6 Astrocytes

Astrocytes are a heterogeneous population of glial cells with many long star-like processes that acquire different morphology and physiological properties depending on the CNS region they populate. Broadly astrocytes are divided into two types, protoplasmic and fibrous (Miller & Raff, 1984). Protoplasmic astrocytes are found in the grey matter extending long processes with which they contact neurones, and endfeet with which they surround blood vessels (Figures 1.1.6.1 and 1.4.1.1). Fibrous astrocytes are found in white matter in close contact with axons and in association with the nodes of Ranvier, projecting longer and thinner processes than those of protoplasmic astrocytes (Butt et al., 1994). Astrocytes *in vitro* can be stained with their specific marker glial fibrillary acidic protein (GFAP), which also stains astrocytes *in vivo* in pathological conditions characterised by gliosis (Bignami et al., 1972).



**Figure 1.1.6.1 Protoplasmic astrocyte in contact with blood vessel**

Two-photon single labelling confocal image of enhanced green-fluorescent protein (eGFP) expressing astrocyte in a cortical slice in contact with a blood vessel. The dense array of processes from a single cell is illustrated. Image adapted from Nedergaard et al., 2003.

Traditionally considered as simply supportive cells, new functions are constantly added to the astrocyte repertoire (reviewed by Nedergaard et al., 2003). Astrocytes are metabolically coupled with neurones. They uptake glucose from the capillaries and convert it glycolytically into lactate, the neuronal metabolic substrate. Lactate is then released by astrocytes and taken up by neuronal cells (reviewed by Tsacopoulos & Magistretti, 1996). Astrocytes define anatomical boundaries in the CNS and one of their major functions is supporting neuronal transmission by maintaining local ion and pH homeostasis (reviewed by Nedergaard et al., 2003). They have functional neurotransmitter receptors and can buffer neurotransmitters such as glutamate or GABA and modulate transmission (Araque et al., 1998; Kang et al., 1998). For

example, elevation of intracellular astrocyte  $\text{Ca}^{2+}$  was shown to potentiate inhibitory transmission in their surrounding neurones (Kang et al., 1998). Astrocytes also regulate the function and integrity of the BBB by surrounding the CNS capillaries with endfeet (see 1.4.1). Another important characteristic of astrocytes is the release of trophic factors such as brain-derived neurotrophic factor (BDNF) and NT-3 that support neuronal and other glial survival (Dreyfus et al., 1999). Astrocytes are implicated in the CNS immune response and were shown to express toll-like receptors TLR1-TLR6 and TLR9 (Carpentier et al., 2005), although TLR-4 expression has been challenged (Lehnardt et al., 2002). Astrocytes release various cytokines such as interleukins, tumour necrosis factor  $\alpha$  (TNF-  $\alpha$ ) and transforming growth factor  $\beta$  (reviewed by Dong & Benveniste, 2001), as well as major histocompatibility complex molecules (MHC) class I (Wong et al., 1984). They are also able to activate T lymphocytes *in vitro* (reviewed by Nair et al., 2008).

When studying the involvement of astrocytes in the immune response the presence of microglia in the experimental system should not be neglected or overlooked. It is possible that in many occasions the cellular responses observed *in vitro* and attributed to astrocytes, might actually be microglial responses (see 1.1.7). For example in the case of nitric oxide (NO) release in cell cultures, a substantial amount of literature exists about iNOS expression and subsequent NO production by astroglial-enriched cultures upon LPS activation of these cultures. Nonetheless it was shown that iNOS appears to be predominantly expressed in microglial cells in mixed glial cultures (reviewed by Saura, 2007), as confirmed by co-expression with a specific microglial marker (Saura, 2007). Some reports have identified iNOS-positive cells as astrocytes by immunostaining them with GFAP, it should be noted though that the morphology of these cells resembles that of microglia *in vitro* (Kong et al., 1996; Korcok et al., 2002).

In response to injury astrocytes are activated, upregulate the expression of intermediate filaments such as GFAP, proliferate and form a dense network of hypertrophic cells, filling the gaps that result from neuronal or glial degeneration. This procedure is called reactive gliosis and forms the glial scar (Reier & Houle, 1988). The glial scar is formed following brain injury or neurodegeneration and is important for controlling damage at the site of injury and suppressing expansion of

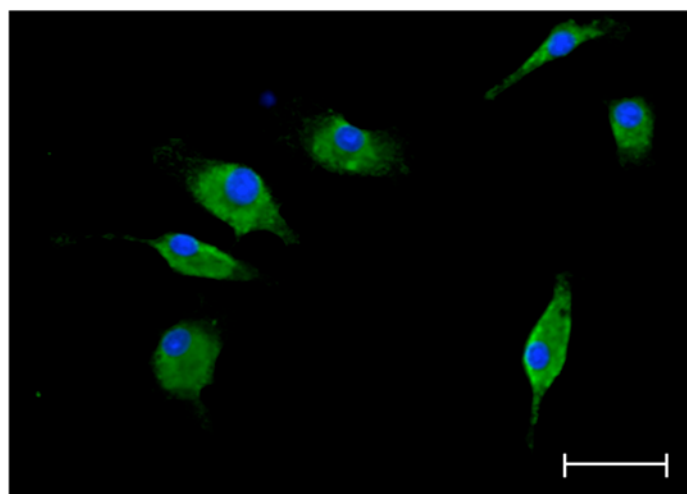
the damage to neighbouring cells. Adverse effects are associated with glial scar formation, since the glial scar is inhibitory for neuronal and precursor cell regeneration, with important implications for trauma and neurodegenerative disease (reviewed by Nair et al., 2008).

Astrocytes have multiple supportive functions in the CNS and a neurological disorder affecting primarily astrocytes would probably be fatal. Nonetheless they can be actively implicated in neurodegenerative disease. For example, it was recently discovered that astrocytes expressing mutant SOD1 could selectively kill wild type motor neurones inducing an ALS phenotype (Nagai et al., 2007). Astrocytes can give rise to astrocytomas, primary CNS tumours that vary from low-grade tumours to glioblastoma multiforme, the most common malignant CNS tumour, with a very poor prognosis (Kleihues et al., 1995). Recently the origin of astrocytomas was challenged and there is evidence that at least some of those tumours might actually derive from neural stem cells (Yuan et al., 2004; Ma et al., 2008).

#### **1.1.7 Microglia**

Microglia are the resident immune effector cells of the CNS. They are small cells present in the brain in a ramified state with highly branched processes. During development and later at postnatal stages, microglia with an amoeboid phenotype derived from the myelomonocytic lineage are produced in the bone marrow, invade the CNS tissue and actively migrate within the brain parenchyma to colonise all regions of the brain (reviewed by Davoust et al., 2008). Amoeboid microglia are also believed to play a role in tissue histogenesis through phagocytosis of inappropriate and superfluous neurones (Marín-Teva et al., 2004) and through promotion of axonal migration and growth (Rakic & Zecevic, 2000). After colonising the CNS, amoeboid cells acquire a ramified phenotype and extend long processes. Traditionally described as ramified or resting, microglia act as sensors of brain pathology, ready to react in response to injury or invasion of a 'foreign' body into the CNS. In contrast to the microglial cell body which remains stable, their processes are highly motile and dynamic *in vivo*, constantly extending and retracting (Davalos et al., 2005; Nimmerjahn et al., 2005). This dynamic movement can be viewed as a

constant survey of the microglial cell's territory, suggesting that the term 'surveying' microglia rather than 'resting' more successfully describes their function in the healthy brain (Pocock & Kettenmann, 2007). Microglia *in vitro* acquire a resting phenotype and can be stained and viewed with macrophage or microglial specific markers (Figure 1.1.7.1).



**Figure 1.1.7.1 Microglia *in vitro***

Fluorescence microscopy image of resting microglia *in vitro*, stained with the specific microglial marker ED1 (green) and DAPI (blue) to view the nuclei. Scale bar = 20 µm.

In response to injury, infection, inflammation or neurodegeneration microglia rapidly gain macrophage morphology, transform into activated cells and migrate to the site of injury (Stence et al., 2001) where they proliferate (Vela et al., 2002). *In vitro* studies of microglial activation, proliferation and subsequent apoptosis are informative of the nature of microglial responses and molecular pathways mobilised (Kloss et al., 1997; Kingham et al., 1999; Hooper et al., 2005). Following activation microglia serve as antigen presenting cells and interact with T lymphocytes by expressing molecules such as class II MHC antigen HLA-DR (Mattiace et al., 1990), CD14 (Rock et al., 2004) and CD40 (Qin et al., 2005). They also secrete a number of inflammatory mediators, such as TNF- $\alpha$  (Sawada et al., 1989; Combs et al., 2001; Taylor et al., 2005), interferon- $\gamma$  (Suzuki et al., 2005) and interleukins (Kloss et al., 1997), which serve to orchestrate the immune response. Other factors secreted by

activated microglia are NO (Kingham et al., 1999), cathepsins (Kingham & Pocock, 2001) soluble Fas ligand (Ciesielski-Treska et al., 2001; Taylor et al., 2005) and the excitatory neurotransmitter glutamate, which is implicated in neurotoxic cascades (Piani & Fontana, 1994).

In response to injury, microglia express most of the toll-like receptors, recognise pathogens and initiate the proinflammatory response. All toll-like receptors (TLR1-TLR9) can be expressed in human microglia (Bsibsi et al., 2002). TLR-4, which is the receptor for LPS, a constituent of the outer cell wall of gram-negative bacteria, was shown to be solely expressed by microglia in the CNS (Lehnardt et al., 2002). Stimulation of microglia with TLR agonists, including LPS for TLR4, peptidoglycan for TLR2, poly(I:C) for TLR3, and CpG DNA for TLR9 leads to increased secretion of most cytokines expressed by microglia and there is evidence for subsequent neurotoxicity in neurodegenerative disease and modulation of the immune response in neuroinflammation (reviewed by Lehnardt, 2009). Interestingly, microglia also express a variety of neurotransmitter receptors, indicative of interplay between microglia and neurones based on the diffusion properties of neurotransmitters released from neurones in a paracrine manner. Microglia possess active  $\alpha$ -amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid (AMPA) and metabotropic glutamate receptors,  $\gamma$ -aminobutyric acid B (GABA<sub>B</sub>), purinergic, adenosinergic, cholinergic, cannabinoid, adrenergic and dopaminergic receptors, as well as receptors for neuroactive peptides. Each neurotransmitter receptor is uniquely involved in microglial function (reviewed by Pocock & Kettenmann, 2007). The expression of metabotropic glutamate receptors by microglia and their potential as therapeutic targets in neurodegenerative disease will be discussed in 1.5.

The question that arises following the review of the substances released and receptors expressed by microglia, is whether activated microglia contribute to neurotoxicity in brain injury, inflammation and neurodegenerative disease, or whether their presence and activation have a neuroprotective effect. In neurodegenerative disease, persistent, chronic microglial activation, accompanied by sustained secretion of inflammatory mediators may exacerbate neurotoxicity and disease processes. In Alzheimer's disease, microglial activation has been described as an early event in disease progression (Cagnin et al., 2001). Amyloid  $\beta$  and secretory

protein chromogranin A (CGA), a protein particularly prominent in the senile plaques associated with Alzheimer's disease, have been shown to activate microglia *in vitro* (Taupenot et al., 1996; Ciesielski-Treska et al., 1998; Kingham et al., 1999; Hooper & Pocock, 2007) and *in vivo* (Xiang et al., 2006). This activation is linked with NO mediated neurotoxicity (li et al., 1996; Qin et al., 2002; Hooper et al., 2009), indicating a toxic role for microglia in disease progression. In Parkinson's disease there is generalised microglial activation, which is not limited to the degenerating regions (Imamura et al., 2003).  $\alpha$ -synuclein aggregates can be internalised by microglia *in vitro* and subsequent microglial activation can result in nicotinamide adenine dinucleotide phosphate (NADPH) oxidase mediated production of reactive oxygen species and neurotoxicity (Zhang et al., 2005). In neuroinflammation, microglia seem to play a dual role, being involved in the initial inflammatory response where they act as antigen presenting and debris phagocytosing cells, but also exhibiting a persistent activation phenotype in disease progression. The involvement of microglia in MS will be extensively discussed in 1.3.4.

Microglia have been implicated in most neurodegenerative diseases, including prion disease, ALS, Huntington's and human immunodeficiency virus (HIV) related dementia (reviewed by Block et al., 2007). Given the progressive and cumulative contribution of microglial activation throughout the course of neurodegenerative disease, monitoring microglial activation could be an indicator of disease progress and severity or could be an early identification marker. At the same time clarifying which microglial processes contribute to neurotoxicity and which have a protective effect could lead to potential therapeutic manipulation of these activation pathways with regulation of the former and enhancement of the latter.

## **1.2 Multiple sclerosis**

### **1.2.1 Epidemiology, symptoms and diagnosis of multiple sclerosis**

Multiple sclerosis (MS) is one of the most common neurological disorders and causes of disability in young adults. Even though people with MS have a near normal life span, more than 60% of diseased individuals will no longer be fully ambulatory 20 years after disease onset with major implications on their life quality. MS is a global disease that has a prevalence of 30 per 100,000 rising to 80 per 100,000 in Europe. The disease is less frequent in non-white individuals and there are geographical patterns to the disease with frequency of MS increasing with distance from the equator. The average age of MS onset is 29.2 years and it has a female predominance of approximately 2:1 (WHO and Multiple Sclerosis International Federation, *Atlas*, 2008).

MS can manifest a variety of neurological symptoms with certain recurring themes and unpredictable course. The most common symptoms are optic neuritis, manifesting unilateral painful loss of vision, double vision, nystagmus, motor symptoms such as muscle weakness, paralysis, spasticity, coordination and balance loss, fatigue, sensory symptoms such as neuropathic pain or paraesthesia, sexual, bowel and bladder dysfunction and cognitive impairment (reviewed by Compston & Coles, 2002). All symptoms have a significant impact on the patient's physical activity and quality of life (Motl et al., 2008). The neurological symptoms of MS are the neurological reflection of acute focal inflammatory demyelinating lesions of the CNS, affecting saltatory conduction at the affected sites. MS is diagnosed based on the the McDonald diagnostic criteria (McDonald et al., 2001; Polman et al., 2005)

The clinical course of MS can follow a varying pattern over time. Relapsing remitting MS (RR-MS) accounts for 85% of all MS cases, with clearly defined relapses followed by full recovery (Lublin & Reingold, 1996). Over years, patients experience episodes of acute worsening of neurological function and develop progressive disability, which is known as secondary progressive MS (SP-MS). During the SP-MS course, lesion activity decreases and degenerative changes predominate over inflammation, leading to progressive brain atrophy (Lublin & Reingold, 1996).



Primary progressive MS (PP-MS), which comprises approximately 15% of MS cases, is progressive from onset with only occasional plateaus and patients become severely disabled within a short period of time (Lublin & Reingold, 1996).

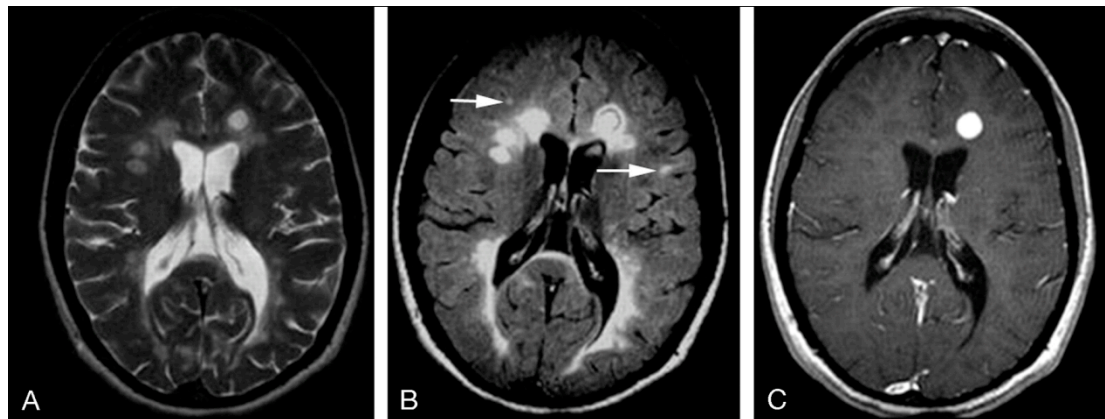
### **1.2.2 Pathophysiology of multiple sclerosis**

From the earliest observations of the disease in 1868 by Jean-Martin Charcot, MS was recognised as an inflammatory process that was associated with focal destruction of myelin sheaths. Indeed the oligodendrocytes are a principal target of the immune attack in MS, where cells of the immune system release cytotoxic cytokines and autoreactive antibodies that target the proteins of the myelin sheath. Subsequent demyelination of the nerve axons results in saltatory conduction compromise, which can lead to the clinical features of MS. Partially demyelinated axons conduct nerve impulses at reduced velocity, while demyelinated axons can discharge spontaneously or show increased sensitivity to mechanical stimuli (reviewed by Compston & Coles, 2002).

The focal sclerotic plaques of MS develop following an acute inflammatory injury. They are characterised initially by acute inflammation, oligodendrocyte and axonal injury, followed by partial or complete recovery, culminating in gliosis and neurodegeneration. Repeated attacks lead to successively less effective remyelination, until a scar-like plaque with preserved axons set in a matrix of astrocytes, an MS lesion, is built up (reviewed by Lassmann et al., 2007). MS lesions can be viewed, and diagnosed, using different MRI techniques (reviewed by Ge, 2006). An example of focal lesions identified with different MRI scan techniques is shown in Figure 1.2.2.1.

Based on histological features and immunohistochemical staining of post-mortem brains, MS lesions can be characterised as active or inactive. Actively demyelinating lesions are identified by the presence of myelin sheaths that can be in the process of dissolution, accompanied by myelin debris, activated infiltrating macrophages and activated resident microglia (Brück et al., 1995). Active plaques are identified at the early stages of the disease, although active components can also be identified in chronic active lesions, where demyelination occurs at the edge of the demyelinated

plaque (Prineas et al., 2001). An inactive, or chronic, demyelinated lesion is characterised by the absence of ongoing destruction of myelin sheaths. Macrophages and microglia can persist in such lesions and gradually clear myelin debris (Brück et al., 1995). The persistent activation of microglia in this environment can have adverse implications for remyelination and neurodegeneration, as will be later discussed (see 1.3.4).



**Figure 1.2.2.1 MRI scans of RR-MS patient revealing focal lesions**

MRI scans of 30-year-old female RR-MS patient. A. T<sub>2</sub>-weighted image. B. Sagittal fluid-attenuated inversion recovery image (FLAIR). The lesions on FLAIR are usually prominent and several small lesions are depicted (arrows). C. Contrast enhanced T<sub>1</sub>-weighted image. The lesion enhancement can be nodule (as shown in this case) or ringlike. Figure adapted from Ge, 2006.

Another histologically defined MS lesion, which due to advances in imaging techniques is gaining interest in the last years, is the preactive lesion. Preactive lesions have been detected in very early cases of MS (Gay et al., 1997; De Groot et al., 2001) as lesion-like signals on MRI scans that immunohistochemically revealed clusters of activated microglia expressing the human leukocyte antigen-DR (HLA-DR) and the microglial marker CD68. They correlated notably with absence of demyelination and leukocyte infiltration. Interestingly, these lesions did not always result in active lesions, suggesting that some level of intrinsic regulation can stop lesion progression. These clearly distinct changes in CNS tissue might be the MS lesion start, as supported by the lack of blood-brain barrier (BBB) damage and inflammation with specific activation of microglia, since there is only little

macrophage infiltration (Gay et al., 1997). It is thus possible that demyelination has not yet occurred. The preactive lesions are defined as round or oval regions demarcated by a cluster of microglia expressing elevated levels of HLA-DR with lack of any detectable demyelination, leukocyte infiltration, gliosis or hypertrophic astrocytes. They are considered as the first specific signs of an emerging MS lesion (van der Valk & Amor, 2009). Another interesting observation is that the two cell types that drive the pathology of these lesions are microglia and oligodendrocytes. It is suggested that soluble factors, such as stress signs released from oligodendrocytes mediate communication between those two cell populations and activate microglia. In a case study, oligodendrocytes in non-demyelinated white matter regions were nitrotyrosine positive and showed early upregulation of the inflammation related gene STAT6 and the myelin proteins MAG and PLP. Neuronal nitric oxide synthase (nNOS) was also upregulated (Zeis et al., 2009). Elevated levels of the small heat shock protein  $\alpha$ B-crystallin were also found in non-demyelinating tissue from MS patients (Sinclair et al., 2005). Exposure of primary microglia to  $\alpha$ B-crystallin results in their activation, iNOS expression, NO production and TNF- $\alpha$  release (Bhat & Sharma, 1999). Additional data suggest that  $\alpha$ B-crystallin is linked to microglial activation in preactive lesions and that its expression is triggered by antibodies to myelin surface proteins. This provides a possible link between preactive lesions and antibodies found in MS (reviewed by van der Valk & Amor, 2009).

Other non-classical lesions have also been identified in MS but they are rare and usually present in atypical disease variants. A note should be made about shadow plaques, a group of somehow optimistic plaques, since they are most probably completely remyelinated ex lesions. They are characterised by reduced density of myelinated fibres associated with thin myelin sheaths (Prineas et al., 1984). In a recent study, 22% of all white matter lesions investigated in post-mortem brains displayed 100% remyelination (Patani et al., 2007). These findings suggest a substantial degree of endogenous lesion repair despite the long disease course and question the idea that remyelination fails early on in the natural history of MS. They could also stand as an exciting therapeutic possibility with the potential of actively invoking such an extensive repair process to the benefit of the patients.

### 1.2.3 Aetiology of multiple sclerosis

MS aetiology remains unknown. Interplay between yet unidentified environmental factors and susceptibility genes appears most likely. MS is characterised by modest heritability and there is a relative risk among siblings, which increases among identical twins (Islam et al., 2006). The heterogeneity of MS points towards common susceptibility genes underlying MS causation, rather than single-gene aetiology.

Genetic linkage studies in multiple-case families have identified the HLA-class II locus within the MHC region on chromosome 6p21.3 to predispose for 20-60% of genetic susceptibility for MS (Haines et al., 1998). The more recently developed genome-wide association studies identified the HLA-DRB1 locus as a risk locus with very strong statistical significance and with 65 different single nucleotide polymorphisms (SNPs) identified (International Multiple Sclerosis Genetics Consortium, 2007; Baranzini et al., 2009). It is still debated whether the genes of interest are within the class II region or extend to the class I region right next to it (reviewed by Oksenberg et al., 2008). HLA-class II genes encode highly polymorphic cell-surface glycoproteins with a fundamental role in immune recognition of self from non-self antigens. Polymorphic HLA-DRB1 residues are associated with human autoimmune disease by affecting the shape and charge of the P4 pocket in the peptide-binding site of the HLA molecule. For example the HLA-DRB\*1501 allele has a large hydrophobic P4 pocket which favours aromatic residue binding to the molecule (Smith et al., 1998). Another candidate gene associated with MS is the IL7R gene. The T244I non-synonymous SNP was identified in the IL7R in the region encoding for one of the transmembrane domains of IL7R protein, with consequences for the function of the receptor. IL7R-mediated signalling is important for T lymphocyte development and survival (Gregory et al., 2007). Two more MS susceptibility SNPs were identified in the IL2RA gene. The IL2-IL2R pathway has an essential role in regulating immune response (Hafler et al., 2007). Finally, in the most recent genome wide association study a role in disease risk was attributed to GPC5, the gene encoding for Glypican 5, a heparan sulfate proteoglycan (Baranzini et al., 2009). An association was found between GPC5 polymorphism and response to IFN- $\beta$  treatment in patients with MS in a successfully replicated pharmacogenomic study

(Byun et al., 2008; Cénit et al., 2009). With the development of high resolution genomic studies more susceptibility gene association studies are expected to emerge in the forthcoming years.

Population genetics alone cannot explain the distribution of MS. The high prevalence rates in northern Europe in contrast to the low frequency rates of the disease in African populations, the latitude effect, or even the association of MS with timing of birth in the year (Willer et al., 2005), all point towards environmental factors contributing to disease occurrence. An explanation for the latitude effect on MS is that sun exposure protects from the disease by raising body levels of vitamin D (Islam et al., 2006). To further support this, direct functional interaction was found between specific recruitment of vitamin D to a vitamin D response element located in the HLA-DRB1\*15 promoter (Ramagopalan et al., 2009). Smoking has also been implicated with the risk of developing MS (Riise et al., 2003). Infectious agents have been implicated as potential environmental risk factors for MS. Such agents include Epstein-Barr virus (EBV), human herpes virus 6, MS-associated human endogenous retroviruses and the bacterial agent *Chlamydia pneumoniae* (reviewed by Giovannoni et al., 2006). EBV is of special interest to MS researchers since it is associated with a dramatic increase in MS risk, which has been obfuscated for many years by the fact that EBV infects more than 95% of the adult population. Individuals who are seronegative for EBV are in very low risk of developing MS, a finding strengthened by studies of paediatric MS (Sumaya et al., 1980; Alotaibi et al., 2004; Pohl et al., 2006). Furthermore, the risk for MS is significantly increased among individuals with a history of infectious mononucleosis, a manifestation of EBV infection in adolescence or adulthood, suggesting that older age of infection with EBV further increases the odds of developing MS (Thacker et al., 2006).

Advances in genetic epidemiology have identified candidate genes as part of the genetic background of MS. These genetic factors can make individuals more 'prone' to MS, but with genetic factors held constant, the disease threshold might be set by the environment. It is most possible that susceptibility to MS is mediated by direct interactions between the environment and genes. Better understanding of the pathophysiological mechanisms underlying MS could clarify the role of candidate genes and point towards the environmental factors responsible for disease initiation.

## **1.3 Cellular pathology and interactions in multiple sclerosis**

### **1.3.1 Immune-mediated oligodendrocyte injury**

The driving force for the inflammatory reaction occurring in the CNS during MS appears to be a T cell mediated immune reaction primarily attacking mature oligodendrocyte myelin proteins. All myelin proteins are potential target antigens for T-cell mediated reactions. They have to be recognised by CD4 lymphocytes and be processed by antigen presenting cells. This mechanism most possibly triggers the inflammatory response, although additional mechanisms are required for myelin sheath destruction (Lassmann et al., 1981). The initial event for the activation of auto-reactive T-cells remains unclear, although a 'molecular mimicry' theory has been proposed, where common microbes can activate antigen presenting cells through the normal acquired immune response but by also expressing cell surface protein sequences that may cross react with self myelin antigens (reviewed by Conlon et al., 1999).

Initial activation of T cells is regulated by dendritic cells in the blood, which are recognised as the primary antigen presenting cells (reviewed by Becher et al., 2006). This is closely followed by proliferation and activation of T cells which via circulation finally adhere to endothelial cells, alter the properties of the BBB and penetrate the CNS (reviewed by Hafler, 2004). T cell infiltration into the CNS depends on alterations in the expression of T cell or endothelial cell surface molecules, together with the secretion of enzymes that degrade the extracellular matrix (Carrithers et al., 2000). Within the CNS, T cells are re-stimulated by microglia and macrophages (Shrikant and Beneviste, 1996; Aloisi et al., 2000). Recent evidence additionally suggests that other factors are also important in MS pathology. Lymphocytes are not always present in early demyelinating lesions (Barnett and Prineas, 2004), and the presence of IgG and complement products on degenerating myelin sheaths in acute lesions suggest an important role for humoral immunity in MS (reviewed by Lucchinetti et al., 1998; Storch et al., 1998). Demyelination follows specific recognition and opsonisation of myelin sheaths by antibodies present on the extracellular surface of myelin membranes. MOG appears to be an important

glycoprotein target for antibody-mediated demyelination (Linington et al., 1988). The destruction of antibody-opsonised myelin can then be mediated by complement (Piddlesden et al., 1993) or activated macrophages (Scolding & Compston, 1991).

Apart from their destruction by specific immunological processes, oligodendrocytes can also be destroyed by toxic molecules released from activated microglia and macrophages. TNF- $\alpha$ , complement, NO and cytotoxic cytokines are only a few of the molecules that can be released from microglia and exhibit direct toxicity to oligodendrocytes and neurones (reviewed by Probert et al., 2000). Exposure of microglia to myelin itself can render them neurotoxic by NO, TNF- $\alpha$  and glutamate release (Pinteaux-Jones et al., 2008). Oligodendrocytes are traditionally considered as cells particularly sensitive to toxic insults such as cytokines, inflammatory molecules, glutamate or complement. There is however evidence pointing towards mature oligodendrocytes being less sensitive than initially considered. *In vitro* they appear to be surprisingly resilient to glutamate (Rosenberg et al., 2003; also see 4.5). The physical presence of other cell populations such as microglia, is necessary for cytokine-induced toxicity to mature oligodendrocytes (see 1.3.5 and 4.4).

Oligodendrocyte pathology is profoundly different between patients or at different stages of disease progression. This observation suggests that the mechanism of demyelination and oligodendrocyte damage is not uniform in MS. Four different patterns of demyelination have been identified, based on the immunopathological nature of the immune response and the patterns of oligodendrocyte injury (Lucchinetti et al., 2000). The basic general pattern of MS lesions (pattern I) describes a T lymphocyte and macrophage dominated immune response characterised by clusters of activated microglia and macrophages in focal demyelinating lesions taking up and digesting myelin debris. This pattern is also characterised by relative axonal sparing and active remyelination. Pattern II resembles pattern I with the additional presence of immunoglobulins (Prineas & Graham, 1981) and activated complement at the active lesions. Tissue damage and axonal loss is also observed. Opsonised myelin can be destroyed by activated macrophages or microglia, or by antibody mediated phagocytosis, pointing towards different immunological mechanisms that can result in oligodendrocyte destruction

and MS phenotype. Pattern III or hypoxia-like lesions are different from the patterns I and II. They exhibit selective MAG loss with preservation of other myelin antigens. The distal processes of oligodendrocytes degenerate first and oligodendrocyte death is apoptotic. These lesions are characterised by hypoxia-like damage and the involvement of microglia is less pronounced (Aboul-Enein et al., 2003). Finally, the less common pattern IV lesions are characterised by severe oligodendrocyte damage that is followed by demyelination with little signs of remyelination. It has been proposed that the subset of patients with these lesions may be genetically predisposed to be more susceptible to immune-mediated damage. Ciliary neurotrophic factor (CNTF) deficiency has been associated with this aggressive type of lesions (Giess et al., 2002). *In vitro*, CNTF protected oligodendrocytes against TNF- $\alpha$  induced death (D'Souza et al., 1996).

### **1.3.2 Axonal demyelination, injury and microglial involvement**

The neurological deficits manifested in MS reflect demyelination, impaired saltatory conduction and axonal injury. Following oligodendrocyte injury electric current is dissipated through formerly myelinated portions of the axon membrane where Na<sup>+</sup> density is low, so conduction is impaired. Additionally, axonal injury and degeneration, which can be acute or chronic is tightly related with structural and functional alterations in MS lesions and it produces the non-remitting persistent neurological defects seen in MS (reviewed by Kornek & Lassmann, 1999).

In demyelinated axons Na<sup>+</sup> channel expression is increased (Foster et al., 1980; Moll et al., 1991), probably as an early compensative mechanism aiming at restoring nerve impulse conduction. But the available evidence suggests that Na<sup>+</sup> channels are important participants in axonal degeneration and CNS axons can be triggered by a sustained Na<sup>+</sup> influx, which drives reverse operation of the Na<sup>+</sup>-Ca<sup>2+</sup> exchanger, an antiporter molecule that can import damaging levels of Ca<sup>2+</sup> into axons (Stys et al., 1992). Based on that finding, data suggest that pharmacological blocking of Na<sup>+</sup> is able to prevent axonal degeneration. Furthermore it was shown that Na(v)1.6 channels which are normally expressed at the nodes of Ranvier, are upregulated and expressed along demyelinated axons and can contribute to axonal injury because



they generate a more persistent current than Na(v)1.2 that normally exist along myelinated axons (reviewed by Waxman, 2006). NO, which is released from activated microglia, is present at increased concentrations in acute MS lesions and injures axons by damaging the mitochondria within them, thereby reducing ATP levels and producing a gradual decrease in Na<sup>+</sup>/K<sup>+</sup> ATPase activity, which limits the ability of axons to extrude Na<sup>+</sup> (Kapoor et al., 2003). Inadequate axonal ATP supply contributes to the degeneration of demyelinated axons in MS because it impairs Na<sup>+</sup>/K<sup>+</sup> ATPase activity, and thereby limits or prevents extrusion of axoplasmic Na<sup>+</sup> (Dutta et al., 2006).

Na<sup>+</sup> channels are also present in microglia (Korotzer & Cotman, 1992; Nörenberg et al., 1994). It was shown that microglia upregulated Na(v)1.6 expression in acute lesions of MS patients. Furthermore when blocking Na<sup>+</sup> channel activity *in vitro*, a 40% reduction in phagocytic activity of cultured microglia was observed and a 75% decrease in the number of inflammatory cells in EAE was seen after treatment with the Na<sup>+</sup> channel blocker phenytoin (Craner et al., 2005). These observations suggest that, in addition to a direct neuroprotective action on axons, Na<sup>+</sup> channel blockers could attenuate axonal injury by a second, parallel mechanism that reduces inflammatory damage through an action on microglia.

It should be noted here that many soluble factors released from microglia have been shown to impair neurones *in vitro* (see 1.1.7). This has implications for acute and chronic MS, since release of such factors contributes to axonal injury, which is associated with irreversible damage in MS (see 1.3.4). Thus, when addressing the issue of neuroprotection in MS or other diseases with a neurodegenerative component, controlling chronic microglial activation may prove important for securing an environment that can sustain and protect neurones.

### **1.3.3 Reduced OPC remyelination ability in multiple sclerosis**

Even though remyelination does take place in demyelinated MS lesions (Patrikios et al. 2006; Patani et al., 2007), its efficiency is limited and it does fail, especially during the chronic phase of the disease. Remyelination efficiency is also affected by age (Shields et al., 1999; Sim et al., 2002), having a profound bearing on MS

progression, which usually lasts a few decades. It has been shown, that even though it can be age-limited (Chari et al., 2003), OPC recruitment in MS lesions does occur and proliferating OPCs are present in demyelinated lesions (Wolswijk, 1998; Solanky et al., 2001). Remyelination failure in MS is more likely due to failure in OPC differentiation than failure in OPC recruitment (Chang et al., 2002) as supported by studies where cells positive for the OPC specific marker NG2 (Chang et al., 2000; Reynolds et al., 2002), and the transcription factors OLIG2 and NKX2.2 (Kuhlmann et al., 2008) were present in MS lesions. An explanation for OPC failure to differentiate into mature myelinating oligodendrocytes in chronic MS lesions is that the lesion environment is inhibitory (see 5.6.3).

In addition to the presence of inhibitory molecules in the lesion environment, the presence of hypertrophic scarring astrocytes might also be inhibitory to remyelination in chronic lesions. OPCs were unable to migrate through astrocyte-rich parenchyma *in vivo* (Franklin et al., 1997). On the other hand, reactive astrocytes present in active lesions have been shown to promote remyelination (Franklin et al., 1991) and express CNTF, a factor that promotes an OPC survival-promoting activity (Albrecht et al., 2007). Active inflammation was also shown to promote remyelination (Setzu et al., 2006). Experimental macrophage depletion in adult rats using clodronate liposomes, which are toxic when injected by macrophages, influenced the growth factor environment, thereby affecting the behavior of OPCs and hence the efficiency of remyelination (Kotter et al., 2005). Finally, active microglia present in the acute lesions are thought to promote remyelination by secreting IL-1 $\beta$  (Mason et al., 2001) and TNF- $\alpha$  that can act through TNFR2 receptors (Arnett et al., 2001). On the other hand IFN $\gamma$  was shown to inhibit remyelination by inducing oligodendrocyte endoplasmic reticulum (ER) stress (Lin et al., 2006).

#### **1.3.4 Microglia in multiple sclerosis**

Different aspects of microglial involvement in MS pathology have already been discussed previously. It is thus clear that microglia have a very important and multi-aspect involvement in MS. Here, the mechanisms by which microglial involvement in

MS can be protective or toxic in different stages of disease progression will be discussed.

Microglia have been implicated in the initial stage of MS. This is supported by evidence showing that in early MS lesion tissue, microglial activation is evident when the myelin sheath as well as the blood-brain barrier are still intact (Gay et al., 1997a). Clusters of activated microglia expressing the human leukocyte antigen-DR (HLA-DR) and the microglial marker CD68 were localised in preactive MS lesions and correlated notably with absence of demyelination and leukocyte infiltration (De Groot et al., 2001). In addition to the immunohistological studies, in a pharmacogenetically induced *in vivo* model of microglial paralysis, the clinical signs of experimental autoimmune encephalitis (EAE) were substantially ameliorated and CNS inflammation was strongly reduced (Heppner et al., 2005). Thus from these studies an important role for microglia in disease initiation is suggested and interestingly MS pathology appears to be initiated from within the CNS parenchyma, independent of an invasive inflammatory cell attack across a compromised blood-brain barrier (BBB) (reviewed by Gay, 2007a).

In the active MS lesions microglia persist in an activated state and are actively involved in the inflammatory response. Microglial activation in active inflammation has been suggested to contribute to the creation of an environment conducive for regeneration. Debris phagocytosis by microglia, such as myelin or blood borne proteins (BBPs) can help clear the lesion and facilitate regeneration especially when T cells infiltrate the lesion and facilitate phagocytosis (Nielsen et al., 2009). Blockade of the microglial phagocytic triggering receptor TREM2 during active EAE resulted in disease exacerbation (Piccio et al., 2007). *In vitro* TREM2 mediated signalling in microglia was shown to facilitate debris clearance (Takahashi et al., 2005). Microglia with myelin debris inclusions have been identified in active MS lesions (Li et al., 1993; Gay et al., 1997). *In vitro*, following myelin phagocytosis microglia secrete cytokines such as IL-6, IL-1 and TNF- $\alpha$  (Williams et al., 1994). TNF- $\alpha$  was shown to massively reduce the amount of myelin ingested by microglia via the complement receptor type 3 (Brück et al., 1992). Furthermore, myelin phagocytosis *in vitro* has been implicated with microglial activation that can lead to neurotoxic cascades (Pinteaux-Jones et al., 2008). It is thus plausible that myelin phagocytosis does

indeed clear the lesion and facilitate regeneration, but at the same time microglia activated by myelin release cytotoxic molecules that exacerbate neurotoxicity.

Cytokine release from microglia is a complex field of research since the plethora of cytokines released from microglia has been implicated in toxic and protective responses. Following LPS activation, microglia secrete TNF- $\alpha$  and FasL and are neurotoxic *in vitro* (Taylor et al., 2005). On the other hand neuroprotective effects have been attributed to microglial secretion of IL-1 $\beta$  (Diem et al., 2003) and IL-23 (Li et al., 2007). Microglia are also activated in a paracrine manner by secreted cytokines. Microglial activation with IFN $\gamma$  was neurotoxic *in vitro* (Butovsky et al., 2006) and inhibited remyelination by inducing oligodendrocyte ER stress (Lin et al., 2006). Microglial activation with IL-4 *in vitro* rendered the cells neuroprotective (Butovsky et al., 2006). TNF- $\alpha$  released from microglia can bind to TNFR1 which is responsible for TNF- $\alpha$ 's apoptotic activity by triggering intracellular events involving a cascade that leads to activation of the transcription factors nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) and c-Jun and activation of caspase 8 (reviewed by Chen and Goeddel, 2002). On the other hand TNFR2 is the TNF- $\alpha$  receptor implicated in neuroprotective cascades (Yang et al., 2002). It was shown that both TNFR1 and TNFR2 mRNA levels were upregulated in cuprizone-induced demyelination. In the cuprizone model of demyelination, copper chelator cuprizone is used in the diet of 8 week old C57BL/6 mice. Mature oligodendrocytes are specifically insulted since they cannot fulfil the metabolic demand of supporting vast amounts of myelin and undergo apoptosis. This is closely followed by microglial recruitment and myelin phagocytosis. If the cuprizone challenge is terminated, an almost complete remyelination occurs within a few weeks (reviewed by Matsushima & Morell, 2001). In this model, microglial activation and cytokine secretion play a pivotal role in cell death, with decreased mitochondrial activity observed in glial cells (Pasquini et al., 2007). In TNF- $\alpha$  knockout mice, cuprizone-induced demyelination and apoptosis were delayed, and remyelination was promoted by OPC proliferation through TNFR2 but not TNFR1 binding of TNF- $\alpha$  (Arnett et al., 2001).

During the chronic, degenerative stages of MS, microglial activation has been attributed to a number of factors that persist in the chronic lesions. BBPs are potential microglial activators. Albumin has been found to activate microglia *in vitro*

and induce calcium response (Hooper et al. 2005; 2009a). Fibrinogen has also been implication in microglial activation by causing proliferation and changes in morphology (Adams et al., 2007). Furthermore, a fibrinogen-derived inhibitory peptide ( $\gamma^{377-395}$ ) attenuated microglia activation and suppressed relapsing paralysis in EAE mice (Adams et al., 2007). Microglia have been shown to strongly express inducible nitric oxide synthase (iNOS) in chronic active MS lesions (Hill et al., 2004) as well as after activation *in vitro* (Hooper et al. 2005; Hooper & Pocock, 2007). NO produced by iNOS reacts to form reactive nitrogen species such as peroxynitrite that can lead to oxidative stress and cytotoxicity. NO has additionally been implicated in BBB disruption in MS (Yamauchi et al., 2007). On the other hand, beneficial immunomodulatory effects in MS have been attributed to NO, so there is still controversy over its role in MS (reviewed by Smith & Lassmann, 2002). To further support the neurodegenerative role of activated microglia in progressive, chronic MS, activated phagocytic microglia were immunohistochemically located at the margins of demyelinated cortical regions (Gray et al., 2008). Previously, direct microglial interaction with oligodendrocytes was demonstrated at the edges of chronic demyelinating lesions (Peterson et al., 2002).

The role of microglia in MS is still under investigation. Secretion of protective mediators, efficient phagocytosis of myelin debris and facilitation of remyelination are beneficial functions of microglia. Although many cytokines such as  $\text{TNF}\alpha$  and  $\text{IFN}\gamma$  are referred as neurotoxic, it seems that their role is rather context-dependent. This elucidates the principal importance of the microglia-mediated stimulation of growth and phagocytic removal of debris in creating a microenvironment for repair and regenerative processes. Elucidating the protective functions of microglia is of increased significance since it can allow for the design of therapeutic strategies targeting those functions and enhancing an already existent protective mechanism.

### **1.3.5 Interactions between microglia and oligodendrocyte lineage cells**

Interactions between microglia and oligodendrocytes have been reported in many cases in MS. For example, it has been proposed that interaction between microglia and oligodendrocytes might be the initial pathological event in MS (see 1.2.2). Thus,

studying the interactions of two cell populations may lead to interesting observations regarding disease pathology.

It was initially documented that although ramified microglia show little contact with oligodendrocytes *in vitro*, following activation, surface binding with oligodendrocytes and myelin phagocytosis increased, especially in the presence of complement (Zajicek et al., 1992). TNF- $\alpha$  production by activated microglia was able to kill oligodendrocytes, although it was far more effective when microglia were in contact with oligodendrocytes (Zajicek et al., 1992) or when oligodendrocytes were exposed to it for a prolonged time period (96 hours; McLaurin et al., 1995). In contrast to that, microglia-derived insulin-like growth factor-2 (IGF-2) reduced oligodendrocyte apoptosis *in vitro*, as well as TNF- $\alpha$  induced oligodendrocyte c-Jun kinase activation, indicating that microglia released soluble factors that differentially affected oligodendrocytes (Nicholas et al., 2002). Insulin-like growth factor-1 (IGF-1) was also found to protect TNF- $\alpha$  induced OPC injury by interrupting the OPC mitochondrial apoptotic pathway (Pang et al., 2007). In a hypoxic rat model, TNF- $\alpha$  and interleukin-1 $\beta$  (IL-1 $\beta$ ) were secreted from microglia as a result of mitogen-activated protein (MAP) kinase pathway activation and this release was coupled with apoptotic mature oligodendrocytes expressing TNFR1 (Deng et al., 2008). TNFR2 on the other hand has been implicated in OPC regeneration and a reparative role for TNF- $\alpha$  following CNS injury was suggested (Arnett et al., 2001). Microglial secreted IL-1 $\beta$  was also implicated in upregulation of late gene expression such as Lcn-2 by oligodendrocytes and expression of lipocalin 2 produced in response to IL-1 $\beta$  could kill or silence the leukocytes mediating the inflammatory response (Howe et al., 2006). IL-1 $\beta$  has also been implicated in astrocyte glutamate buffering disruption in a mixed glial environment, resulting in oligodendrocyte excitotoxic death (Takahashi et al., 2003).

It is strongly suggested that non-activated microglial conditioned medium promotes OPC survival and maturation into oligodendrocytes (Pang et al., 2000; Nicholas et al., 2001; Miller et al., 2007). When microglia are activated with LPS, it has been proposed that LPS activation does not only abolish the protective effect of microglial conditioned medium on OPCs, but furthermore when LPS activated microglial conditioned medium is added to OPC cultures, it induces OPC death (Pang

et al., 2000). This is also true for co-cultures of microglia and immature oligodendrocytes (Li et al., 2008). The effect of LPS on OPCs was shown not to be cell autonomous but mediated through activation of microglial cells via the TLR4 receptor (Lehnardt et al., 2002). The same study also determined that only LPS activated microglia and not astrocytes or fibroblasts significantly mediated OPC death when grown in Boyden chambers (chambers that allow separate culture of the cell populations in close proximity). Astrocytes in contact with immature oligodendrocytes were again unable to induce cell toxicity in response to LPS (Li et al., 2008). In the study of Pang et al. LPS activated astrocyte conditioned medium did not induce oligodendrocyte death, although the ability of conditioned medium to promote OPC survival and proliferation was abolished.

Quiescent microglial conditioned medium was shown to enhance mature oligodendrocyte survival (Pang et al., 2000). LPS activated microglia were also shown to be beneficial for cell survival when co-cultured with mature oligodendrocytes (Miller et al., 2007). In a different study, LPS activated microglia co-cultured with mature oligodendrocytes for 48 hours were found to induce mitochondrial membrane depolarization and cell death in oligodendrocytes (Domercq et al., 2007). Both an AMPA receptor antagonist and a reactive oxygen species (ROS) scavenger were able to reverse this toxicity. Blocking the system  $x_c^-$  cystine-glutamate antiporter in oligodendrocytes, which uptakes cystine in exchange for intracellular glutamate, also reduced toxicity. Mature oligodendrocytes, even if not to the same extent as OPCs, were found to be sensitive to NO and demonstrated a deep and rapid decrease in ATP levels (Baud et al., 2004). In an earlier study mature oligodendrocytes were also found to be sensitive to NO donors and exhibit a  $Ca^{2+}$  influx and EGTA protection in response to NO generated by the NO donor S-nitroso-N-acetylpenicillamine (SNAP) (Boullerne et al., 2001).

In an early study the effect of the NO donor SNAP on glia was measured by mitochondrial depolarisation and cell viability. It was concluded that oligodendrocytes were much more sensitive to NO-mediated toxicity than astrocytes or microglia (Mitrovic et al., 1994). It was also proposed that microglial mediated oligodendrocyte death correlated with NO production (Merrill et al., 1993). It should be noted however that in that study oligodendrocyte death was achieved only with

25:1 microglia:oligodendrocyte ratio in culture, a ratio much higher than *in vivo*, that may only represent conditions of sustained microglial activation and proliferation. In a recent study on human primary oligodendrocyte cultures it was suggested that NO alone was unlikely to induce oligodendrocyte toxicity, whereas its by-product peroxynitrite was toxic to MAG positive oligodendrocytes (Jack et al., 2007). In a rat cell culture study peroxynitrite was found responsible for LPS mediated cell death of immature oligodendrocytes when co-cultured with microglia (Li et al., 2005). Interestingly, it was later shown that in mixed cell cultures where astrocytes were also present, inhibition of iNOS or peroxynitrite decomposition catalysis did not prevent immature oligodendrocyte death (Li et al., 2008). It was suggested that although astrocytes may not be required for LPS toxicity their presence changes the death mechanism to a NO and peroxynitrite independent mechanism. The effects of NO and its byproduct peroxynitrite on oligodendrocyte lineage cells are summarised in Table 1.3.5.1. It may be possible that astrocytes protect against peroxynitrite toxicity due to their high antioxidative capacities (Peuchen et al., 1997). It was determined that the induced immature oligodendrocyte death in those mixed cultures was not due to excitotoxicity or a caspase dependent pathway but was caused by TNF- $\alpha$  and IFN $\gamma$  in combination when glial cells were present (Li et al., 2008). OPC and mature oligodendrocyte death *in vitro* has to be carefully translated. It is evident that the presence of additional cell populations has an important effect on microglial - oligodendrocyte communication and can alter cellular responses.

In an *in vivo* study where OPCs were transplanted into the spinal cord of myelin-defective mutant rats, OPCs only survived when microglia were not fully activated, or when animals were pre-treated with minocycline (Zhang et al., 2003). Minocycline is an antibiotic that prevents oligodendrocyte death by inhibiting microglial p38 mitogen-activated protein kinase (MAPK) phosphorylation, RhoA activation and p75 neurotrophin receptor expression after spinal cord injury (Yune et al., 2007). In the cuprizone model of demyelination, inhibition of microglial activation with minocycline prevented cuprizone-induced demyelination (Yune et al., 2007). The cuprizone model can be useful when investigating interactions between microglia and oligodendrocytes.



In view of the current literature, it becomes apparent that interactions between microglia and oligodendrocytes depend on the microglial activation state, the presence of other cell types, the balance of soluble factors secreted and the developmental stage of oligodendrocytes. Careful translation of previous research and design of adequate experimental strategies will help establish a view as to how microglia contribute to oligodendrocyte cell death in MS.

<b>NO or ONOO<sup>-</sup> source</b>	<b>Cells affected</b>	<b>Insult</b>	<b>Reference</b>
DTP/NO or DETA/NO	Immature oligos /mature oligos	ATP level decrease/cell death - peroxynitrite independent	Baud et al., 2004
SNAP or SNP	Mature oligos	Ca <sup>2+</sup> influx/cell death	Boullerne et al., 2001
SNAP	GalC <sup>+</sup> oligos	Mitochondrial damage /Cell death	Mitrovic et al., 1994
ONNO <sup>-</sup>	Mature oligos	Cell death /SNAP independent	Jack et al., 2007
Microglia	Immature oligos	Cell death	Merill et al., 1993
Microglia	Immature oligos	ONNO <sup>-</sup> mediated cell death	Li et al., 2005
Microglia	Immature oligos	ONNO <sup>-</sup> independent cell death (astrocytes present in culture)	Li et al., 2008

**Table 1.3.5.1 The effect of NO and its by-product peroxynitrite on oligodendrocyte viability**

The NO donors dipropylenetriamine-NONOate (DPT/NO), diethylaminetriamine-NONOate (DETA/NO), S-nitroso-N-acetylpenicillamine (SNAP) sodium nitroprusside (SNP) or peroxynitrite (ONOO<sup>-</sup>) were used to study the effects of NO on oligodendrocyte (oligo) viability.

### **1.3.6 Astrocyte involvement in multiple sclerosis**

Astrocytes are the most abundant cell type of demyelinated plaques, contributing to the pathogenesis of MS by forming a glial scar with highly filamentous processes expressing GFAP, nestin and vimentin (Holley et al., 2003). The glial scar aims at preventing further tissue damage but it also prevents tissue regeneration. It was

shown that OPCs were unable to migrate through astrocyte-rich CNS parenchyma *in vivo* (Franklin et al., 1997; Bannerman et al., 2007). At the site of lesion astrocytes are thought to inhibit myelination by secreting FGF-2 (Messersmith et al., 2000) and hyaluronan which accumulates in demyelinated MS lesions and inhibits OPC maturation (Back et al., 2005). Astrocytes also express molecules that inhibit axonal regeneration. Astrocyte-derived chondroitin sulphate proteoglycans (CS-PGs), neurocan (Asher et al., 2000) and brevican (Yamada et al., 1997) are up-regulated around glial scars and inhibit axonal regeneration *in vivo* (Quaglia et al., 2008).

Natalizumab, an  $\alpha 4$ -integrin monoclonal antibody, inhibits the interaction between T cells and endothelium or astrocytes and reduces infiltration of inflammatory cells into the CNS. It is used for MS therapy and strongly reduces brain lesion number and relapses in patients (Polman et al., 2006). In EAE, astrocytes and endothelial cells, express vascular cell adhesion molecule-1 (VCAM-1), the T cell receptor  $\alpha 4$ -integrin ligand required for the T cells to traffic past the BBB and enter the CNS (Rosenman et al., 1995). Thus, astrocytes might be equally important with endothelial cells in T cell infiltration (reviewed by Williams et al., 2007). Additionally, at the astrocyte endfeet, matrix metalloproteinases (MMPs) facilitate immune cell extravasation by breaking down extracellular matrix and tight junctions. It was found that suppression of MMP activity in astrocytes reduced immune cell trafficking into the CNS and attenuated EAE (Dubois et al., 1999).

Cytokine and growth factor release from astrocytes has also been demonstrated in MS. Astrocyte conditioned medium can promote OPC survival through the action of PDGF, leukemia inhibitory factor (Gard et al., 1995), NT-3, insulin-like growth factors, IL-4 and CNTF (Barres et al., 1993). BDNF is also expressed by astrocytes in active MS lesions (Stadelmann et al., 2002). It has been proposed that astrocytes are implicated in the postulated neuroprotective effect of TNF- $\alpha$  in MS by expressing BDNF in a TNF- $\alpha$  dependent manner (Saha et al., 2006). In human MS tissue astrocytes were shown to express MHC class I (Höftberger et al., 2004) and class II (Zeinstra et al., 2000) although other studies showed absence of MHC class II expression in astrocytes (Bö et al. 1994; Höftberger et al., 2004). IL-12, IL-23 (Constantinescu et al., 2005) and IL-17 (Tzartos et al., 2008) are also secreted by astrocytes, supporting their involvement in T cell presentation and activation.

Astrocytes are implicated in MS not only by forming the glial scar but also by expressing growth factors and cytokines, as well as responding to such molecules in a paracrine manner. They take active part in the molecular concert that mediates the immune response and the lesion formation in MS and the balance of secreted molecules determines if a toxic or protective phenotype will prevail. The involvement of astrocytes in the immune response in MS, as well as their contribution in supporting an inhibitory environment for regeneration in the chronic phases of disease progression are areas that could be further investigated.

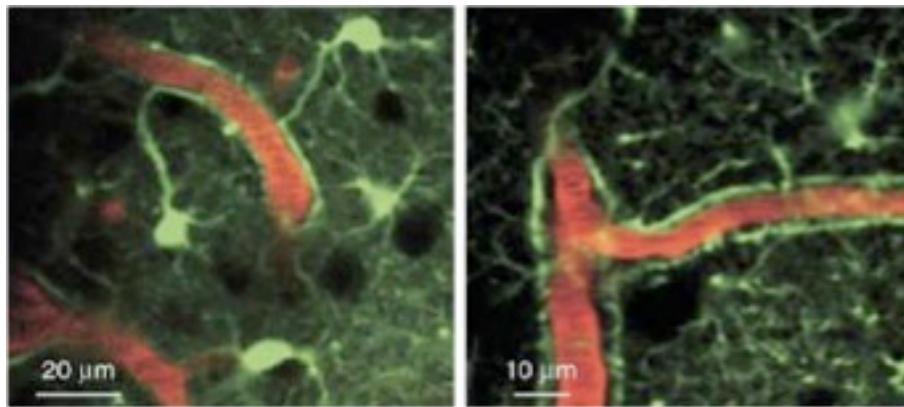
## **1.4 The blood-brain barrier**

### **1.4.1 Physiological structure and function of the blood-brain barrier**

The blood-brain barrier (BBB) is a physiological barrier at the interface between the blood and the brain tissue, formed by endothelial cells of blood vessels that enter the brain (Reese & Karnovsky, 1967). The BBB maintains the microenvironment of the brain and controls homeostasis. It ensures a constant supply of nutrients such as oxygen and glucose for brain cells but also protects the brain from external insults such as chemicals and pathogens. The BBB guides leukocytes to migrate into the brain, an immune surveillance event important for antigen recognition, known as trans-endothelial migration (reviewed by Weiss et al., 2009). Important for the recognition and adhesion of leukocytes are the endothelial adhesion molecules inter-cellular adhesion molecule-1 (ICAM-1), VCAM-1 (Oppenheimer-Marks et al., 1991) and platelet endothelial cell adhesion molecule-1 (PECAM-1; Muller et al., 1993). The BBB endothelial cells differ significantly from non-brain endothelial cells (reviewed by Persidsky et al., 2006) and form tight junctions (Brightman & Reese, 1969; van Deurs & Koehler, 1979) that consist of the tight junction specific molecules occludin (Furuse et al., 1993), claudin-1, claudin-5 (Liebner et al., 2000) and junctional adhesion molecules (JAMs), specifically important for BBB development and leukocyte recruitment (Del Maschio et al., 1999).

Astrocytes and neurones are also involved in the BBB. Astrocytes envelop 99% of the BBB endothelium (Figure 1.4.1.1) and it has been suggested that they play an important role in maintaining BBB tightness and function (Tao-Cheng et al., 1987). Astrocytes *in vivo* were able to induce BBB characteristics to endothelial cells (Janzer & Raff, 1987) and glial-derived neurotrophic factor (GDNF) secreted by astrocytes enhanced BBB function and tight junction integrity (Igarashi et al., 1999). It was also shown that *src*-suppressed C-kinase substrate (SSeCKS) stimulated expression of angiopoietin-1 in astrocytes, resulting in BBB differentiation and tight junction formation (Lee et al., 2003). A different study showed that the BBB could self-repair in the absence of astrocytes (Willis et al., 2004), suggesting that astrocytes may modulate the BBB phenotype without a direct involvement in BBB formation and

restoration. It was recently shown that angiotensin II released from astrocytes acted on angiotensin receptors on endothelial cells and contributed towards BBB integrity by acting upon occludin organisation (Wosik et al., 2007). Neurones are also found in close contact with the brain endothelium, and although the molecular mechanisms of neuronal input to the BBB are still under investigation, it is well established that high levels of regional neuronal activity co-localise with increased blood flow, a process known as neurovascular coupling (reviewed by Leybaert, 2005).



**Figure 1.4.1.1 Astrocytic ensheathment of blood vessels**

Two-photon fluorescence images of astrocytes and blood vessels in rat neocortex, stained using the astrocyte-specific marker sulforhodamine (SR101, green) and tail-vein injection of dextran (red), respectively. Astrocytic endfeet ensheath almost the entire cerebrovascular surface. Figure adapted from Nimmerjahn et al., 2004.

#### **1.4.2 Blood-brain barrier disruption in multiple sclerosis**

In MS, BBB alteration is a key factor for disease progression. BBB opening can be visualised using MRI and monitoring the diffusion of gadolinium through endothelial tight junctions (reviewed by Waubant, 2006). Early events such loss of the BBB solute barrier (Kermode et al., 1990) and enhanced trans-endothelial leukocyte and monocyte infiltration (reviewed by Minagar & Alexander, 2003) point towards a role for BBB in early acute, active and inflamed lesions. Expression of class II MHC in endothelial cells during relapses has also been reported (van der Maesen et al., 1999), although endothelial cell class II MHC expression has been discounted by other studies (Bö et al., 1994; Ulvestad et al., 1994).

There is a large amount of data showing altered levels of endothelial proteins in MS. It is suggested that endothelial cells are activated by cytokines released by leukocytes and in active MS lesions molecules promoting trans-endothelial infiltration such as ICAM-1, VCAM-1, e-selectin and PECAM-1 are upregulated (Washington et al., 1994). In EAE occludin dephosphorylation correlated with disease symptom initiation (Morgan et al., 2007). Claudin-3 selective loss was also reported in active lesion EAE tissue (Wolburg et al., 2003). The vascular endothelial growth factor (VEGF) released from astrocytes was consistently upregulated in acute and chronic MS lesions, acting upon the BBB and altering its function by lowering the expression of junction proteins (Proescholdt et al., 2002). Proinflammatory cytokines released from macrophages and microglia in response to inflammation can affect tight junction molecules. IFN- $\gamma$  in particular, was shown to downregulate occludin expression (Oshima et al., 2001), whilst in turn IFN- $\beta$  upregulated occludin (Minagar et al., 2003), explaining in part the therapeutic effect of IFN- $\beta$  administration to MS patients. The effect of NO release in MS lesions is still unclear because NO production has been implicated in both toxic and protective processes. eNOS expressed by endothelial cells induces vasodilation, while iNOS expressed by microglia and macrophages in response to stress produces NO. NO can form reactive nitrogen species, such as peroxynitrite, that have been implicated in BBB disintegration (Boje & Lakhman, 2000). In EAE, BBB alterations were prevented by the peroxynitrite scavenger uric acid, limiting disease severity (Kean et al., 2000).

BBB opening in MS is one of the most consistent features of the disease. Monocyte and T cell infiltration into the brain is accompanied by blood borne soluble proteins (BBPs) that are physiologically excluded from the CNS. Such proteins are microbial antigens (Odoardi et al., 2007), complement and immunoglobulins (Gay & Esiri, 1991), albumin (Sokrab et al., 1988; Butter et al., 1991; Kevil et al., 1998), fibrinogen and fibrin (Sokrab et al., 1988; Wakefield et al., 1994; Gay et al., 1997), fibronectin (Sokrab et al., 1988) and interleukin 15 (Pan et al., 2008). In Alzheimer's disease, the 'opening' of the BBB results in deposition of serum proteins such as albumin (Alafuzoff et al., 1983), fibrinogen and fibrin (Lipinski & Sajdel-Sulkowska, 2006), that can alter the endothelial tight junctions and acting upon cells of the nervous system such as microglia resulting in neurotoxicity (Hooper et al., 2005; 2009).

### **1.4.3 Therapeutic interventions by targeting the blood-brain barrier**

The BBB as a physical barrier between the blood circulation and the brain can be manipulated to therapeutically address disease. There is much interest on how BBB disturbance in disease can be constrained, or how inflammatory responses that lead to trans-endothelial migration can be limited in inflammatory and neurodegenerative disease. For example, current MS treatment with IFN- $\beta$  is beneficial by controlling trans-endothelial migration of activated leukocytes into the CNS (Minagar et al., 2003). Natalizumab, an  $\alpha$ 4-integrin antibody, is currently an approved therapy for controlling relapsing MS. It aims at impairing leukocyte and monocyte trafficking into the brain by blocking their adhesion to VCAM-1 and mucosal addressin cell adhesion molecule-1 (MAdCAM-1) on BBB endothelial cells (reviewed by Johnson, 2007).

Extensive research is focused on the manipulation of the BBB to allow drug delivery into the brain. This can be achieved by transiently increasing BBB permeability or by modulating the expression of specific BBB transporters (reviewed by Weiss et al., 2009). The transient BBB 'opening' is achieved by intracarotid infusions of hyperosmotic solutions, followed by administration of drugs, viral vectors or nanoparticles. There is though an increased risk of toxic side effects (reviewed by Rapoport, 2000).

In conclusion, BBB pathology should not be neglected when studying brain disease. 'Opening' of the BBB, followed by increased inflammatory response, changes in BBB endothelial cell properties and efflux of blood components into the brain could lead to secondary neurotoxic cascades that could exacerbate disease progression.

## 1.5 Microglial metabotropic glutamate receptors as therapeutic targets

### 1.5.1 Classification and characteristics of metabotropic glutamate receptors

Glutamate, the most abundant excitatory neurotransmitter in the brain, binds to two types of receptors, the ionotropic glutamate receptors (iGluRs) and the metabotropic glutamate receptors (mGluRs). The iGluRs are multimeric ligand gated ion channels that allow ions such as  $\text{Na}^+$  and  $\text{Ca}^{2+}$  to enter the cell. iGluRs are subdivided into three groups, N-methyl-D-aspartic acid (NMDA) receptors, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and kainate (KA) receptors (reviewed by Tikhonov & Magazanik, 2009). The mGluRs are metabotropic receptors that couple to G-proteins to modulate slow synaptic transmission through intracellular second messengers. Thus far, eight different mGluR receptors have been identified, mGluR1 to mGluR8. mGluR receptors have been classified into three different groups (groups I-III) based on their sequence similarity, pharmacological properties and signal transduction mechanisms (reviewed by Benarroch, 2008). Group I consists of the mGluR1 and mGluR5 receptors. Group I receptors are coupled to phospholipase C and subsequently to the hydrolysis of phosphatidylinositol into inositol triphosphate ( $\text{IP}_3$ ) and diacylglycerol (DAG), leading to intracellular  $\text{Ca}^{2+}$  release and PKC activation respectively. Group II consists of the mGluR2 and mGluR3 receptors. Group II receptors are negatively coupled to the cyclic adenosine monophosphate (cAMP) cascade in the cell. Group III consists of the mGluR4, mGluR6, mGluR7 and mGluR8 receptors. Group III receptors are also negatively coupled to adenylyl cyclase (reviewed by Benarroch, 2008). The characteristics of all groups of mGluRs are summarised in Table 1.5.1.1. Glutamate has different affinity for different mGluRs, as summarised in Table 1.5.1.2. Thus mGluR subtypes are differentially activated by extracellular glutamate concentrations.



Group	Receptor	Transduction mechanism	Location	Effect
I	mGluR1	Activation of PLC leading to IP <sub>3</sub> receptor mediated intracellular Ca <sup>2+</sup> release and DAG mediated activation of PKC	Postsynaptic (peripheral to active zones)	Facilitation of NMDA mediated responses
	mGluR5	Potentialiation of L-type Ca <sup>2+</sup> channels		Increase in neuronal excitability
		Inhibition of K <sup>+</sup> channels		Promotion of LTP or LTD
		G-protein independent phosphorylation cascades		
II	mGluR2	Inhibition of AC	Presynaptic (preterminal region)	Inhibition of neurotransmitter release
	mGluR3	Inhibition of voltage-gated Ca <sup>2+</sup> channels	Postsynaptic	Decrease in neuronal excitability
		Activation of K <sup>+</sup> channels		
III	mGluR4	Inhibition of voltage-gated Ca <sup>2+</sup> channels	Presynaptic terminal(active zone)	Inhibition of neurotransmitter release
	mGluR6	Activation of K <sup>+</sup> channels		
	mGluR7			

PLC = phospholipase C; PKC = protein kinase C; LTP = long-term potentiation; LTD = long-term depression; AC = adenylyl cyclase.

**Table 1.5.1.1 Characteristics of mGluR receptor groups I-III**

Table adapted from Benarroch, 2008.

mGluR	EC <sub>50</sub> (μM) for glutamate
mGluR1a	9-13
mGluR5a	3-10
mgluR2	4-20
mGluR3	4-5
mGluR4a	3-20
mGluR6	16
mGluR7	1000
mGluR8	0.02

**Table 1.5.1.2 Potency (EC<sub>50</sub>) of glutamate for different mGluR subtypes**

Potency (EC<sub>50</sub>) in μM of glutamate for different mGluR subtypes expressed in cell lines, adapted from Conn & Pin, 1997.

### 1.5.2 Microglial metabotropic glutamate receptors

All brain cell types express different mGluRs. Depending on their location within the CNS, neurones express all three groups of mGluRs (Byrnes et al., 2009a). Stimulation of group I mGluRs in neurones increases neuronal excitability (Zhong et al., 2000) and group I mGluR antagonists have been shown to be neuroprotective (Faden et al., 2001). Astrocytes express the group I receptor mGluR5 (Balázs et al., 1997; 1998; Cai et al., 2000) and the group II receptor mGluR3 (Aronica et al., 2003) although their expression decreases during development. mGluR5 expression can be upregulated by growth factors *in vitro* (Miller et al., 1995), or increased following kainate induced epileptic seizures (Ulas et al., 2000). Group III mGluR4 and mGluR8 receptors were only found in reactive astrocytes in MS lesions (Geurts et al., 2005). OPCs were shown to express mGluR1, mGluR5, mGluR2, mGluR3 and mGluR4a. mGluRs were downregulated by day 6 *in vitro* with only traces of mGluR5 detected in mature oligodendrocytes (Deng et al., 2004). A different study showed mGluR3 to be constitutively expressed in OPCs and mature oligodendrocytes, whilst mGluR5 was downregulated (Luyt et al., 2006).

Microglia express functional mGluRs of all three groups. Group I mGluR5a receptor mRNA was first identified (Biber et al., 1999) and protein expression was later confirmed (Byrnes et al., 2009b). Group II mGluR2 and mGluR3 receptors (Taylor et al., 2002) and group III mGluR4, mGluR6 and mGluR8 receptors (Taylor et al., 2003) are also expressed in microglia. Direct stimulation of mGluR2 receptors in microglia can contribute to microglial neurotoxicity through release of TNF- $\alpha$  in concert with microglial derived soluble Fas ligand (Taylor et al., 2005). On the other hand, direct stimulation of group III mGluRs reduces microglial reactivity and neurotoxicity (Taylor et al., 2003). These data demonstrate that it is likely that strategies regulating specific mGluR activation may have a therapeutic effect in neurodegenerative disease where microglial activation is involved.

Activation of microglia with LPS, amyloid  $\beta$  or CGA peptide, all expressed in Alzheimer's plaques, caused microglial glutamate release and resulted in a feedback activation of group II mGluRs, thus fuelling microglial neurotoxicity (Taylor et al., 2002). On the contrary, activation of microglial group III mGluRs did not occur,

suggesting that more glutamate may need to be released to trigger group III mGluRs. It has been shown that group II mGluRs can respond to lower glutamate levels than group III mGluRs do (Pin & Duvoisin, 1995; Table 1.5.1.1). Since group III mGluR receptors can be neuroprotective following LPS, CGA or amyloid  $\beta$  microglial activation (Taylor et al., 2003), group III mGluRs offer further targets for controlling microglial toxicity in neurodegenerative disease (reviewed by Pocock & Kettenmann, 2007). It was also shown that treatment with group I receptor mGluR5 agonists inhibited LPS induced microglial activation and NO production via PLC and PKC activation and  $\text{Ca}^{2+}$  release (Byrnes et al., 2009b).

### **1.5.3 Metabotropic glutamate receptors as therapeutic targets in MS**

mGluR receptors are recognised as promising therapeutic targets for many neurological diseases where glutamatergic disturbance is evident (reviewed by Marek, 2004). Drugs acting at mGluR receptors can regulate the glutamatergic system without affecting the normal synaptic transmission. In MS patients, glutamate levels in the CSF are elevated during relapses or in patients with severe neurological defect (Stover et al., 1997; Barkhatova et al., 1998). Glutaminase, the enzyme that converts glutamine to glutamate, as well as glutamate transporter elevated levels correlated with axonal damage in MS tissue (Werner et al., 2001). In MS, excitotoxicity contributes to neuronal death (reviewed by Gonsette, 2008) and OPC or developing oligodendrocyte death (Figure 4.5.1). Manipulation of the mGluR response to elevated glutamate levels may thus be beneficial for cell survival.

Immunohistochemical staining of active MS lesions revealed upregulation of mGluR5, mGluR2 and mGluR3 in astrocytes and microglia (Geurts et al., 2003). Group III mGluR8 receptors were also upregulated in the microglial population in active MS lesions and to a lesser extent in chronic lesions. mGluR4 and mGluR8 were expressed by a subpopulation of astrocytes in the rim of chronic lesions (Geurts et al., 2005). In a different immunohistochemical study of active MS lesions mGluR1 receptors were upregulated in astrocytes and microglia, as well as dystrophic axons. Group II receptors were stained in astrocytes and bipolar cells that the authors speculated were OPCs (Newcombe et al., 2008). Microglial mGluRs can induce

toxicity or neuroprotection (see 1.5.2) and it can be speculated that group II microglial and astrocyte receptor activation in acute lesions is triggered by elevated glutamate levels, thus contributing to microglial toxicity. On the other hand upregulation of astrocytic mGluR4 and mGluR8 receptors at the rim of the MS lesions might indicate high levels of glutamate, activating group III mGluRs in astrocytes able to respond in a similar way to microglial group III receptors, attenuating neurotoxicity (Taylor et al., 2003). The neuroprotective effect of group III mGluRs can also be confirmed by the reverse of neuronal cell death induced by myelin activated microglia by a group III mGluR agonist (Pinteaux-Jones et al., 2008).

The role of microglial mGluRs in oligodendrocyte toxicity in MS as well as other neurodegenerative diseases is an interesting field of investigation, since the observations on neurones point towards a regulatory role for mGluR receptors in MS. In *in vitro* studies of OPC cells it was shown that group I mGluR activation was able to reverse kainic acid induced OPC toxicity (Kelland & Toms, 2001) or hypoxic-ischemic developing oligodendrocyte injury (Deng et al., 2004). On the other hand mature oligodendrocytes have only been shown to express mGluR3 (Luyt et al., 2006). Thus if a regulatory role for microglial mGluRs in oligodendrocyte toxicity is revealed, therapeutic avenues directly targeting microglia, OPCs or neurones could be developed.

## 1.6 Aims and objectives

This project aimed to investigate interactions between microglia and cells of the oligodendrocyte lineage in MS. To achieve this, the experimental strategies designed aimed to:

- Establish an *in vitro* model for the study of microglial-oligodendrocyte interactions by culturing the cell populations separately or in contact.
- Examine LPS and BBPs as potential microglial activators in MS and elucidate the intracellular pathways that lead to microglial activation and cytokine release.
- Examine the effect of resting and activated microglia on the survival of oligodendrocyte cells of different maturation stages.
- Examine the effect of BBPs on oligodendrocyte and neuronal survival, and its regulation by microglial manipulation.
- Elucidate the toxic effect of glutamate to oligodendrocyte lineage cells.
- Examine how stimulation or inhibition of specific microglial mGluR receptors could affect survival and maturation of oligodendrocytes or their precursors.

This study yields information about how microglial activation can be protective or toxic to cells of the oligodendrocyte lineage, depending on the nature of the microglial activators and the developmental stage of the oligodendrocyte cells. It also investigates the effect of BBPs on oligodendrocyte maturation and survival of oligodendrocytes and neurones. The toxic effect of BBPs in MS can be controlled by microglial activation or by blockade of oligodendrocyte intracellular pathways. Manipulation of cells and modulation of molecular pathways that prevent oligodendrocyte maturation or promote cell death can lead to novel strategies to prevent oligodendrocyte and neuronal death, thus slow disease progression in people with MS.

## **2. MATERIALS AND METHODS**

## 2.1 Materials

Sprague Dawley pups were obtained from Central Biological Services, UCL, London, UK and were killed by cervical dislocation and decapitation in accordance with the United Kingdom Animals (Scientific Procedures) Act, 1986. The BV-2 cell line was a kind gift from Dr Claudie Hooper (MRC Centre for Neurodegenerative Research, Institute of Psychiatry, Kings College London, UK) and was originally obtained from Dr FS Tzeng (Department of Life Sciences, National Cheng Kung University, Taiwan). The CG-4 (Central Glia-4) cell line was a kind gift from Professor Elek Molnar and Dr Karen Luyt (Department of Anatomy, MRC Centre for Synaptic Plasticity, University of Bristol, School of Medical Sciences, Bristol, UK). Foetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium (DMEM) powder (52100-039), Minimum Essential Medium (MEM) powder (11700-077), fungizone (15290026) and Trizol reagent (15596018) were obtained from Invitrogen (Paisley, UK). Earle's balanced salts solution (EBSS) was obtained from Life Technologies (Paisley, UK). Tissue culture plasticware were obtained from Triple Red (Buckinghamshire, UK) or VWR (Leicestershire, UK) and 13 mm coverslips (MIC3336) were obtained from Scientific Laboratory Supplies (Nottingham, UK). 175 cm<sup>2</sup> tissue culture flasks (353112) were obtained from Marathon (London, UK). CO<sub>2</sub> gas cylinders were obtained from BOC gases (Guilford, UK). Phosphate buffered saline (PBS) powder was purchased from ICN Biomedicals (Maidenhead, UK). 30% w/v acrylamide (20-2100-05) and N, N, N', N'-tetramethylethylenediamine (TEMED, 20-3000-25) were obtained from Severn Biotech (Worcestershire, UK). ECL Plus Western Blotting Reagents (RPN2106) and Amersham Hyperfilm ECL (28-9068-37) were purchased from GE Healthcare (Buckinghamshire, UK). The antibodies rabbit anti-NG2 (sc-20162), goat anti-COX-2 (sc-1746), goat anti-GFAP (sc-6171) donkey anti-goat-IgG (sc-2056), goat anti-rabbit-IgG (sc-2004) were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Donkey anti-mouse-IgG antibody was purchased from Affinity Bioreagents (Cambridge, UK). Mouse anti-ED1 (MCA341) and mouse anti-CD11b (OX-42, MCA275EL) antibodies were obtained from Serotec (Oxford, UK). Rabbit anti-iNOS antibody (#610333) was obtained from BD Transduction Lab (Oxford, UK). Rabbit anti-MBP antibody (AB980) and Re-Blot Plus (2504) were purchased from

Chemicon (Watford, UK). Mouse anti-MOG antibody (NCI-031) was purchased from Sera-Lab (Sussex, UK). Mouse anti-phospho-p42/44 MAPK (9106) and rabbit anti-total MAPK (9102) antibodies were obtained from Cell Signalling Technology (New England Biolabs, Hertfordshire, UK). Y-27632 (Calbiochem 688000) was purchased from VWR (Leicestershire, UK). N-(2,6-dioxo-3-piperidinyl)phthalimide (Thalidomide, 0652), 2-Amino-5,6-dihydro-6-methyl-4H-1,3-thiazine hydrochloride (AMT-HCl, 0871), (S)-3,5-Dihydroxyphenylglycine (DHPG, 0805), (RS)-1-Aminoindan-1,5-dicarboxylic acid (AIDA, 0904), 6-Methyl-2-(phenylazo)-3-pyridinol (SIB-1757, 1215), (2S,2'R,3'R)-2-(2',3'-Dicarboxycyclopropyl) glycine (DCGIV, 0975), (RS)-1-Amino-5-phosphonoindan-1-carboxylic acid (APICA, 1073), L-(+)-2-Amino-4-phosphonobutyric acid (L-AP4, 0103), (RS)-4-Phosphonophenylglycine (RSPPG, 1220) and (S)-2-Amino-2-methyl-4-phosphonobutanoic acid (MAP4, 0711) were purchased from Tocris (Bristol, UK). TNF- $\alpha$  Quantikine ELISA kit (#RTA00) was purchased from R&D Systems (Oxford, UK). Fibroblast growth factor (bFGF, 100-18B) and platelet-derived growth factor (PDGF, 100-13A) were obtained from Peprotech (London, UK). Limulus Amebocyte Lysate (LAL) Pyrochrome test was purchased from Associates of Cape Cod Incorporated (Liverpool, UK). LPS (L2762), FV albumin (A6272), FITC conjugated albumin (A9771), fibrinogen (F4883), fibrin (F5386), mouse anti- $\beta$ -actin clone AC-15 (A5441), N-acetylaspartylglutamate (NAAG, A5930), glutamic acid monosodium salt (G1626), Bradford protein assay reagent (B6916), Immobilion P polyvinylidene difluoride (PVDF) membranes (P2938), Tween-20 (P7949), ammonium persulfate (APS, A3678), developer (P7042), fixer (P7167), 4',6-diamidino-2-phenylindole (DAPI, D8417), 2'-(4-Ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5'-bi-1H benzimidazole (Hoechst 33342, B2261), propidium iodide (P4170), FITC-isolectin B<sub>4</sub> (L2895), DNAase (D5025), soybean trypsin inhibitor (T9003), bovine trypsin (T9935), porcine trypsin (T4424), cytosine arabinofuranoside (AraC, C1768), Holo-Transferrin (T1283), triiodothyronine (T3, T0281), thyroxine (T4, T1775), sodium selenite (S5261), insulin (I6634), biotin (B4639), hydrocortisone (H0135), progesterone (P8783), putrescine (P5780), poly-D-lysine (PDL, P1149), accutase (A6964), freezing medium (C6164), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, H0891), ampicillin (A2804), gentamicin (G1397), kanamycin (K0254), penicillin-streptomycin solution (P4333) and all bench grade chemicals were obtained from Sigma (Dorset, UK).



## **2.2 Cell lines**

### **2.2.1 The BV-2 Cell Line**

The BV-2 cell line is a murine cell line generated by infecting primary microglial cell cultures with the v-raf/v-myc oncogene carrying retrovirus J2 (Blasi et al., 1990). It retains most of the morphological, phenotypical and functional properties of primary microglia and has been widely used as a tool in microglial cell research.

BV-2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal bovine serum (FBS) supplemented with 2 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in 6% CO<sub>2</sub>. BV-2 cells were harvested with accutase (Invitrogen), which was then neutralized with an equal volume of medium. The cells were centrifuged at 3645 g for 5 minutes at 19°C (Eppendorf Centrifuge 5804R), resuspended in fresh warm medium and seeded into a new sterile flask. To cryopreserve them, BV-2 cells were frozen into Sigma freezing medium and stored at -80°C. They were thawed by warming up the frozen vial and pouring its content into BV-2 medium into a culture flask, placing in the incubator (37°C, 6% CO<sub>2</sub>) and replacing the medium the following day.

BV-2s were routinely plated on 13 mm sterilised glass coverslips in 24 well plates at a density of 20,000 cells/well or on 35 mm 6 well plates at a density of 100,000 cells/well. At least two hours before activating the plated BV-2 cells, the medium was substituted for serum free medium (DMEM, 2 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin). BV-2 cells in serum free media were more ramified and responded better to activating stimuli. BV-2 conditioned medium was used to incubate cells of the CG-4 cell line and its toxic or protective effect on those cells after a period of 24 or 48 hours was assessed using the live-dead cell staining and immunostaining methods described in 2.5 and 2.6. The viability of BV-2 cells plated on coverslips following incubation with specific activators, agonists, antagonists or inhibitors was also assessed by live-dead cell staining. Expression of iNOS, a marker of microglial activation was studied by immunostaining. Cells plated on 6 well lysate dishes at a density of 100,000 cells/well were used for the western blotting assay, which is described in detail in 2.7.

### 2.2.2 The CG-4 Cell Line

CG-4 is an oligodendrocyte precursor cell (OPC) permanent cell line, established in 1992 (Louis et al., 1992). CG-4 cells were initiated from dissociated cerebral cortex mixed glial cultures of newborn Sprague-Dawley rats. CG-4 cells are small, motile, process-bearing cells which proliferate actively. CG-4 cells were grown in modified Sato medium supplemented with 30% conditioned medium from the B104 neuronal cell line (B104-CM) at 37°C in 6% CO<sub>2</sub> (Luyt et al., 2006). B104 cells were previously grown and medium was collected and frozen at -80°C by Dr Fleur Pinteaux-Jones, Cell Signalling Laboratory, Institute of Neurology, UCL. Modified Sato medium consisted of Dulbecco's modified Eagle's medium (DMEM), 0.1% (w/v) bovine serum albumin fraction V, 60 µg/L progesterone, 16.1 mg/L putrescine, 5 µg/L sodium selenite, 400 µg/L tri-iodothyronine, 400 µg/L l-thyroxine, 50 mg/L holo-transferrin, 5 mg/L insulin, 2 mM l-glutamine, 5 mg/L basic fibroblast growth factor (bFGF) (Peprotech), 1 mg/L platelet-derived growth factor (PDGF) (Peprotech), 100 U/ml penicillin, 100 mg/ml streptomycin 100 ng/ml kanamycin and 50 ng/ml gentamycin. B104-CM provided the OPCs with growth factors, such as PDGF and b-FGF, although for controlled culture conditions supplementary growth factors were added in the medium. The combination of PDGF and bFGF is able to override the timing mechanism responsible for OPC differentiation and to induce sustained self renewal of OPCs (Bögler et al., 1990). It has been suggested that CG-4 cells can be cultured in serum free and B104-CM free medium to induce differentiation, where 98% of the CG-4 cells should differentiate into oligodendrocytes within two days (Louis et al., 1992). Attempts to do that were not successful. Furthermore, there are indications that differentiated CG-4 cells are not a reliable model for mature oligodendrocyte studies due to incomplete differentiation (Itoh et al., 2000). Culturing CG-4 cells in medium with (5-20) % fetal calf serum (FCS), resulted in them differentiating into type-2 astrocytes (Louis et al., 1992). Type-2 astrocytes acquire a stellate morphology and express A2B5 (OPC specific antigen), as well as GFAP (astrocyte specific antigen). These cells are not thought to exist *in vivo* (Fulton et al., 1992).

CG-4 cells were cultured on flasks, culture dishes or coverslips coated with 100 mg/L poly-D-Lysine (PDL). Cells were passaged every 2-3 days. Care was taken to prevent the cells from growing until confluent, because they then differentiated and

eventually died. They also should not be split too sparse because that delayed proliferation, resulting in an increased number of differentiated cells (cells differentiated spontaneously over time). Cells were harvested using porcine trypsin (2.5 mg/ml in DMEM) and were observed under the microscope until they started to detach (usually in less than 1 minute). Trypsin inhibitor (0.05 mg/ml in DMEM) was added and the cells were centrifuged at 720 g for 4 minutes at 19°C (Eppendorf Centrifuge 5804R). The cells were then resuspended in Sato medium supplemented with 30% B104 conditioned medium (CG-4 medium) and split between two 75cm<sup>2</sup> flasks. The flasks were swirled gently to ensure that the cells were evenly distributed onto the flask surface. Otherwise, cells in dense areas proliferated faster and started differentiating, whilst proliferation was delayed when cells were sparsely spread out. The medium was changed every two days.

CG-4 cells were plated on 100 mg/L PDL coated 13 mm glass coverslips at 20,000 cells/well. The following day the medium was replaced. Old medium was removed carefully using a pipette, because the cells did not adhere strongly on the coverslips. The cells were ready to use from the second day on. BV-2 cell conditioned medium was added to the cells at a 1:1 ratio and cell death was assessed. To freeze the cells, after trypsinisation and centrifugation as normal, they were resuspended into 2 mls of DMEM with 20% FBS and 10% DMSO, split between two vials, placed in a propan-2-ol containing box and frozen at -80°C. The cells were thawed by rapidly defrosting in Sato medium with 20% FBS and centrifuging through this at 720 g for 4 minutes. They were then resuspend in Sato medium and centrifuged again at 720 g for 4 minutes to wash the FBS away. Finally they were resuspended in Sato medium and supplemented with 30% B104 conditioned medium (CG-4 medium).

## 2.3 Primary cell cultures

### 2.3.1 Primary culture of mixed glia

To obtain primary microglial and oligodendrocyte cultures, the widely used shaking method for primary oligodendrocytes (McCarthy & de Vellis, 1980; Armstrong, 1998) was used with modifications. The method was optimised for the isolation of microglia as well as oligodendrocytes. Initially a mixed glial culture was obtained, from which, by differential shaking, the microglia and oligodendrocytes could be isolated.

5-6 day old Sprague-Dawley rat pups were killed by cervical dislocation and decapitation in accordance with the United Kingdom Animals (Scientific Procedures) Act, 1986. Brains were quickly removed and placed into a sterile petri dish containing minimum essential medium (MEM) with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 100 U/ml penicillin, and 100 mg/ml streptomycin. The hemispheres were carefully dissected and placed into a new petri dish with fresh MEM-HEPES, where they were mechanically dissociated by triturating through a 19-gauge, a 23-gauge and a 25-gauge needle until the dissociated tissue was homogenous and without any lumps. The homogenate was then transferred to a 50ml tube of MEM-HEPES and centrifuged at 230 g for 10 minutes. The loose pellet acquired was resuspended in prewarmed medium (DMEM, 10% FBS, 1 mM sodium pyruvate, 25 mg/ml gentamycin). The cells were then seeded into tissue culture flasks coated with 100 mg/L PDL. The equivalent of 2 brains was seeded per 75 cm<sup>2</sup> flask and the cells were placed in an incubator at 37°C in 6% CO<sub>2</sub>. After 3 days *in vitro* the medium was gently replaced with fresh, prewarmed medium with as less disturbance of the cell growing surface as possible. The cells were thereafter fed every 3 days. After 10 days in culture the growing surface was confluent with an astrocyte monolayer. On top of that microglia and clusters of cells (oligodendrocyte precursor cells, OPCs) could be easily distinguished. This indicated that the mixed glial cultures were ready to shake and differentially dissociate the different cell populations.

If primary mixed glial cultures were desired, the cells were initially seeded directly onto coverslips rather than tissue culture flasks, by adding 25  $\mu$ l of the cellular suspension (1 ml of medium/brain) on each coverslip. After 20 minutes 500 ml of prewarmed DMEM-10% FBS medium was added to the cells and as with the flasks, the medium was carefully replaced after 3 days *in vitro*.

### **2.3.2 Isolation of primary microglia**

On day 12 *in vitro* the flasks with mixed glia were shaken to remove microglia. After feeding with fresh media, the flasks were sealed well with Parafilm and placed on an orbital shaker at 37°C to be shaken at 125 rpm for 5 hours. The supernatant with the dislodged microglial cells was then collected and centrifuged at 230 g for 5 minutes at 19°C (Eppendorf Centrifuge 5804R). The cells were resuspended in DMEM-10% FBS, counted and plated on 13 mm glass coverslips in 24 well plates at a density of 50,000 cells/well or on 35 mm 6 well lysates plates at a density of 200,000 cells/well. The flasks (with astrocytes and OPCs remaining on the growing surface) were fed with fresh medium and placed in the incubator until ready for further use.

### **2.3.3 Isolation of primary oligodendrocytes**

After microglial removal, the flasks were ready to be shaken to obtain the OPCs. The flasks were sealed again and shaken for approximately 18 hours (overnight) on the orbital shaker at 190 rpm at 37°C. If after shaking small round cells were still observed on top of the astrocyte layer under the microscope, the flasks were shaken for a few more hours. Finally the supernatant with the dislodged cells was centrifuged at 230 g for 10 minutes (Eppendorf Centrifuge 5804R). The pellet was resuspended in 3 ml DMEM-10% FBS and seeded onto an uncoated 100 mm sterile Petri dish. This was left in the incubator for 2 hours at 37°C in 6% CO<sub>2</sub>. During that time any contaminating astrocytes and microglia adhered tightly to the dish surface, while OPCs only loosely adhered. The medium was then gently aspirated and expelled 4 or 5 times to remove any loosely adhered OPCs from the Petri dish. The dish surface was checked under the microscope to make sure that all the small round cells (OPCs) were removed. The OPC containing medium was then centrifuged

at 230 g for 10 minutes at 19°C (Eppendorf Centrifuge 5804R) and the pellet was resuspended in 1ml of prewarmed DMEM-10% FBS. The cells were counted and plated onto 100 µg/L PDL coated 13 mm glass coverslips in 24 well plates at a density of 25,000 cells/well or onto 35 mm dishes at a density of 200,000 cells/dish. Cells were seeded onto each coverslip (100 µl/coverslip) or dish (2ml/dish) and left for approximately 20 minutes. After 20 minutes 400ml of defined serum free medium were gently added in each well. The defined medium used to culture oligodendrocytes consisted of DMEM (with NaHCO<sub>3</sub>), 0.1% BSA, 1mM sodium pyruvate, 5 µg/ml insulin, 50 µg/ml transferrin, 30 nM selenium, 400 µg/ml tri-iodothyronine (T3), 5 ng/ml biotin, 10 nM hydrocortisone, 0.5% FBS, 25 µg/ml gentamycin, 100 U/ml penicillin and 100 mg/ml streptomycin. The medium was changed after 24 hours and from then on every 2 days. FBS could be increased to 2% after the first 4 days, but if many contaminating microglia and astrocytes were present FBS helped their survival. To maintain OPCs in an undifferentiated state 5 mg/L basic fibroblast growth factor (bFGF) and 1 mg/L platelet-derived growth factor (PDGF) were added to the medium. For experiments on OPC cells the medium was changed the following day and cells were ready to use.

#### **2.3.4 Isolation of microglial – oligodendrocyte lineage co-cultures**

Alternatively, to obtain mixed cultures of microglia and OPCs, the differential adhesion step described above was omitted and cells were plated straight onto PDL coated (100 µg/ml) 13 mm glass coverslips (25 x 10<sup>3</sup> cells) or 35 mm lysate dishes (2 x 10<sup>5</sup> cells). The co-cultures initially consisted of 3:2 MG/OPCs and after oligodendrocyte maturation, the ratio decreased to 2:3, as assessed by *Bandeiraea simplicifolia* isolectin B<sub>4</sub> (BSI-B<sub>4</sub>) staining (see 3.4).

#### **2.3.5 Primary culture of cerebellar granule cells**

Cerebellar granule cells (CGCs), are small (10 micrometres in diameter) excitatory neurones found within the granular layer of the cerebellum. CGCs receive excitatory input from mossy fibres and release glutamate (Gallo et al., 1982). CGCs have been extensively used for neurotransmission (Pocock et al., 1993; Pocock et al., 1995) and

neurotoxicity studies (Kingham & Pocock, 2001; Pinteaux-Jones et al., 2008; reviewed by Suñol et al., 2008).

To obtain CGCs the cerebella from 5-6 day old Sprague-Dawley rats were used (Courtney et al., 1990; Kingham et al., 1999). The rat pups were killed by cervical dislocation and decapitation in accordance with the United Kingdom Animals (Scientific Procedures) Act, 1986. Cerebella were quickly removed and placed into a Petri dish containing ice cold 100 mM phosphate buffer saline (PBS), 0.3 % bovine serum albumin (BSA), 10 mM glucose and 0.38%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ . This solution was referred to as solution B and was further used as a base to prepare the solutions needed for the cell preparation. Cerebella were mechanically dissociated with a flamed sterile razor blade and the resulting suspension was added into a tube with 0.5 mg/ml trypsin in solution B. Cells were placed at 37°C for 10 minutes and the effect of trypsin was then neutralised by adding an equal volume of solution B with 8 units/ml DNAase and 8 µg/ml soybean trypsin inhibitor. The suspension was then centrifuged at 65 g for 5 minutes. The solid pellet was resuspended in a few (4) ml of solution B with 50 µg/ml soybean trypsin inhibitor and 50 units/ml DNAase. The pellet was triturated with three fire-polished glass pipettes of decreasing diameter to form a homogeneous suspension. The suspension was slowly added on top of an aliquot of Earle's balanced salts solution (EBSS) to form a layer. This was centrifuged at 100 g x 5 minutes. The soft pellet was then resuspended in 1 ml of culture medium consisting of minimum essential medium (MEM) with  $\text{NaHCO}_3$ , 30 mM glucose, 1 mM glutamine, 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 6 µg/ml ampicillin. The volume of the suspension was adjusted and cells were plated on 100 µg/ml PDL coated 13 mm coverslips at a density of 800,000 cells per coverslip. 100 µl of the suspension were added onto every coverslip in 24 well plates and placed in the incubator (37°C) for 1 hour. After 1 hour, 500 µl of culture medium were added into the wells. The cultures were maintained at 37°C with 6%  $\text{CO}_2$ . After 24-36 hours the cells were washed and half the culture medium was replaced with fresh medium supplemented with 10 µM cytosine arabinofuranoside (AraC) to prevent glial cell proliferation. The CGCs were ready to use after 7 days in vitro.

## 2.4 Cell treatment

Primary microglia and BV-2 cells were routinely cultured for 24 hours but no longer before use. Before activating the cells, the medium was changed to serum free medium and cells were left for at least 2 hours before activation. Microglia or BV-2s were activated with lipopolysaccharide (LPS), fraction V albumin (FV albumin), fibrinogen (FG) or fibrin (FN) for 24 hours. The TNF- $\alpha$  synthesis inhibitor thalidomide and the iNOS inhibitor AMT-HCl were added 1 hour prior to activation. The ROCK inhibitor Y-27632, TLR4 antibody and CD11b antibody were added 24 hours prior to addition of microglial activators. All compounds used to treat primary cell cultures and cell lines are listed in Table 2.4.1. The concentrations selected are justified in the corresponding result chapters and were either selected based on previous studies or selected following titration of the proteins or inhibitors used. After 24 hours of treatment the microglial medium was collected and centrifuged at 10,500 g for 2 minutes at room temperature to remove any floating cells or debris and rapidly frozen and stored at -20°C. Agonists and antagonists of the mGluR receptors of microglial cells were used in conjunction with the above activators (added 1 hour in advance to assure their binding to the receptors before the activators were introduced to the cells) or alone. All the mGluR agonists and antagonists used are listed in Table 2.4.2. The concentrations of the mGluR agonists or antagonists used in this study were based on previous studies on the effect of mGluR manipulation on microglia (Taylor et al., 2002; 2003; 2005).

CGCs or cells of the oligodendrocyte lineage were treated directly with proteins or inhibitors of interest or with microglial conditioned medium at a ratio of 1:1 in their culture medium. For such experiments microglia were cultured in CGC medium (for CGCs) or in serum free DMEM (for oligodendrocytes). After 24 or 48 hour incubation with microglial conditioned medium cells were used for live dead staining, immunostaining or western blotting.



Compound	Description	Concentration	Catalogue No.
<b>LPS</b>	Prototype microglial activator	0.1-0.5 µg/ml	Sigma L2762
<b>FV albumin</b>	Microglial activator	1 mg/ml	Sigma A6272
<b>FG</b>	Microglial activator	2.5 mg/ml	Sigma F4883
<b>FN</b>	Microglial activator	1 mg/ml	Sigma F5386
<b>Thal</b>	TNF-α synthesis inhibitor	3 - 10 µg/ml	Tocris # 0652
<b>AMT-HCl</b>	iNOS inhibitor	1 - 3 mM	Tocris # 0871
<b>Y-27632</b>	ROCK inhibitor	50 mM	Calbiochem 688000
<b>Anti-TLR4</b>	TLR-4 blocking antibody	3 µg/ml	Santa Cruz sc-13591 L
<b>Anti-CD11b</b>	CD11b blocking antibody	10 µg/ml	Serotec MCA27EL

**Table 2.4.1 Activators, inhibitors and antibodies administered to primary cultures**

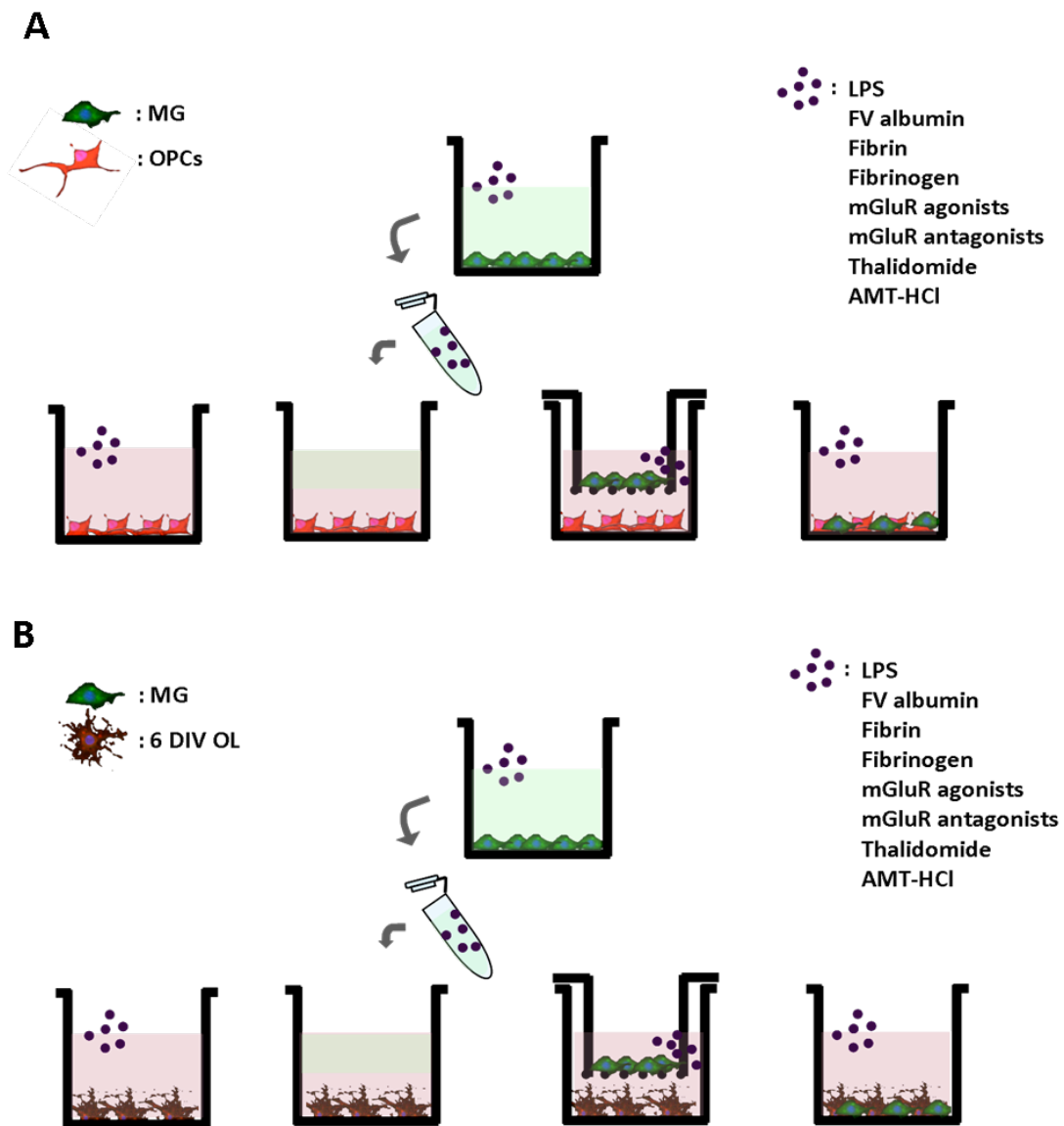
Lipopolysaccharide (LPS), FV albumin, fibrinogen (FG), fibrin (FN), thalidomide (Thal), 2-Amino-5,6-dihydro-6-methyl-4H-1,3-thiazine hydrochloride (AMT-HCl), Y-27632, toll-like receptor 4 antibody (anti-TLR4) and CD11b antibody (anti-CD11b) were administered to primary cell cultures at concentrations justified in the text.

Compound	Description	Concentration	Catalogue No.
<b>DHPG</b>	Group I mGluR agonist	100 mM	Tocris # 0805
<b>AIDA</b>	Group I mGluR antagonist	250 mM	Tocris # 0904
<b>SIB-1757</b>	mGlu5 antagonist	50 mM	Tocris # 1215
<b>DCGIV</b>	Group II mGluR agonist	500 nM	Tocris # 0975
<b>NAAG</b>	mGlu3 agonist	50 mM	Sigma A5930
<b>APICA</b>	Group II antagonist	200 µM	Tocris # 1073
<b><sub>L</sub>-AP4</b>	Group III mGluR agonist	100 mM	Tocris # 0103
<b>RSPPG</b>	Group III mGluR agonist	100 mM	Tocris # 1220
<b>MAP4</b>	Group III mGluR antagonist	500 mM	Tocris # 0711

**Table 2.4.2 Metabotropic glutamate receptor (mGluR) agonists and antagonists**

(S)-3,5-dihydroxyphenylglycine (DHPG), (RS)-1-aminoindan-1,5-dicarboxylic acid (AIDA), 6-methyl-2-(phenylazo)-3-pyridinol (SIB-1757), (2S,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl) glycine (DCGIV), N-acetylaspartylglutamate (NAAG), (RS)-1-amino-5-phosphonoindan-1-carboxylic acid (APICA), L-(+)-2-amino-4-phosphonobutyric acid (<sub>L</sub>-AP4), (RS)-4-phosphonophenylglycine (RSPPG) and (S)-2-amino-2-methyl-4-phosphonobutanoic acid (MAP4) were administered to primary cell cultures at concentrations based on previous studies on the effect of mGluR manipulation on microglia (Taylor et al., 2002; 2003; 2005).

For the line of experiments where interactions between primary microglia and oligodendrocytes were studied, oligodendrocytes were treated with microglial conditioned medium as described above, or the two cell populations were cultured together. Microglia were either cultured in 0.4  $\mu\text{m}$  pore size cell culture inserts (Falcon, 3495) that were fitted into the culture wells of 24 well plates, or the two cell populations were co-cultured onto the same coverslip at a ratio of approximately 3:2 MG/OPCs, which decreased to approximately 2:3 after oligodendrocyte maturation. Microglial activators or other compounds were applied directly to the monocultures, co-cultures, or the wells with the cell inserts. The cell inserts allowed free diffusion of medium and proteins secreted from the cells, whilst the cell populations were restricted into their compartments without physical contact. The same set of experiments was repeated for OPCs and 6 DIV mature oligodendrocytes. The experimental rationale is depicted in Figure 2.4.1.



**Figure 2.4.1 Experimental rationale for the study of microglial - oligodendrocyte interactions**

To study primary microglial (MG) - oligodendrocyte (OL) interactions, microglial activators, mGluR agonists and antagonists and specific inhibitors were added directly to oligodendrocytes, directly to microglia and then microglial conditioned medium was transferred to oligodendrocytes, to co-cultures where microglia were cultured in 0.4  $\mu\text{m}$  pore size cell culture inserts at a density of 20,000 cells/insert above oligodendrocytes (25,000 cells/well), or to co-cultures of the two cell populations onto the same coverslip at a ratio of approximately 3:2 MG/OPCs, or 2:3 MG/6 DIV OL. The same set of experiments was repeated for microglia and OPCs (A) or for microglia and 6 DIV mature oligodendrocytes (B).

## 2.5 Live cell staining

### 2.5.1 Live-dead staining

Live-dead cell staining was performed on primary microglia, primary oligodendrocyte lineage cells, co-cultures, CGCs, as well as on BV-2s or CG-4s. The fluorescent nuclear dyes 2'-(4-Ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5'-bi-1H benzimidazole (Hoechst 33342) and propidium iodide (PI) were utilised. Hoechst 33342 is a membrane permeable dye that binds to all nuclei by binding to the minor groove of DNA (Portugal & Waring, 1988). It fluoresces blue with excitation wavelength at 345 nm and emission wavelength at 478 nm and is used to assess cell viability (Bonfoco et al., 1995). Apoptotic nuclei stain a brighter blue than healthy nuclei and also tend to be smaller, punctuate and possibly fragmented. PI is a red fluorescent dye with excitation wavelength at 536 nm and emission wavelength at 617 nm that binds to DNA. It is membrane impermeant so only enters and binds to cells when the cell membrane is permeable, in early necrotic death or late stage apoptosis (Crissman et al., 1976). The percentage of red stained cells in a cell population was thus the percentage of dead cells in the cell population. When studying co-cultures containing microglia, cells were also stained with 2 µg/ml FITC conjugated *Bandeiraea simplicifolia* isolectin B<sub>4</sub> (BSI-B<sub>4</sub>). BSI-B<sub>4</sub> is a lectin with exclusive affinity for the α-D-galactosyl oligosaccharide residue (Wood et al. 1979) and it selectively binds to microglia in the brain (Streit & Kreutzberg 1987). FITC conjugated BSI-B<sub>4</sub> selectively stained microglia green and allowed them to be distinguished from oligodendrocytes when counting the cells (see 3.3.2 for details).

Following treatment, the cells were incubated with 1 mg/ml propidium iodide (PI) (Morgan et al. 2004) and 0.6 mg/ml Hoechst 33342 for 15 minutes in 37°C (Kingham et al., 1999). 2 µg/ml FITC conjugated BSI-B<sub>4</sub> for 2 hours were also added if microglia were present. The coverslips with live, unfixed and unpermeabilised cells were then placed on a slide with basic medium (3 mM NaCl, 3.5 mM KCl, 0.4 mM KH<sub>2</sub>PO<sub>4</sub>, 20 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid, 1.2 mM Na<sub>2</sub>SO<sub>4</sub> and 1.3 mM CaCl<sub>2</sub> at pH 7.4) and were viewed under the microscope. The percentage of cell death in each coverslip was then calculated. In co-cultures microglia stained

green with BSI-B<sub>4</sub> were manually counted and distinguished from oligodendrocytes. Thus, live and dead cells of each cell population could be manually counted and calculated separately, so the differential effect of treatments to microglia and oligodendrocytes could be assessed.

The microscope used to view the cells was a fluorescence microscope (Zeiss Axioskop 2, Oberkochen, Germany). Cells were viewed at excitation 343 nm with emission 480 nm for Hoechst 33342 and at excitation 581 nm with emission 596 nm for PI and at excitation 495 nm with emission 517 nm for FITC conjugated BSI-B<sub>4</sub>. Images of 3 or 4 fields per coverslip were captured using a Zeiss AxioCam HRc camera. The lenses used were a 20X or a 40X Plan-NEOFLUAR. Live and dead cells on each field were counted with Image J 1.42i software, or manually where required. In every experiment three coverslips for each condition studied were used. All live-dead experiments were repeated in triplicate.

### **2.5.2 FITC conjugated albumin uptake**

To assess internalisation of albumin by microglia, primary microglia were incubated with 1 mg/ml FITC conjugated albumin for 0, 30, and 60 minutes at 37°C. Cells were also incubated with 1 mg/ml FITC conjugated albumin for 60 minutes at 4°C to prevent internalisation and to ensure that the observed fluorescence was not due to surface binding (Tabernero et al., 1999; Hooper, 2003; Pinteaux-Jones, 2007). Microglia were then washed one time in PBS to remove any FITC conjugated albumin remaining in the media and were viewed under the microscope on a slide with basic medium (3 mM NaCl, 3.5 mM KCl, 0.4 mM KH<sub>2</sub>PO<sub>4</sub>, 20 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid, 1.2 mM Na<sub>2</sub>SO<sub>4</sub> and 1.3 mM CaCl<sub>2</sub> at pH 7.4) as in 2.5.1. Cells were viewed at excitation 343 nm with emission 480 nm for Hoechst 33342 and at excitation 495 nm with emission 517 nm for FITC conjugated albumin. Images of 3 or 4 fields per coverslip were captured using a Zeiss AxioCam HRc camera. The lenses used were a 20X or a 40X Plan-NEOFLUAR.

## 2.6 Immunostaining

Immunostaining of primary microglia, primary oligodendrocytes and BV-2 cells was performed to assess cell culture purity (see Chapter 3) or to identify the expression of labelled proteins in the cells. The method was applied on fixed cells, which were permeabilised for the identification of intracellular proteins (Taylor et al., 2002; Taylor et al., 2003).

Cultured cells were fixed on glass coverslips in 4% paraformaldehyde at 4°C. After 30 minutes the cells were permeabilised in 100% ice-cold methanol for 20 minutes at -20°C, followed by 3 washes in phosphate buffer saline (PBS). To block non-specific binding sites the cells were incubated for 30 minutes at room temperature in 4% normal goat or horse serum solution in PBS, depending on the antibody host. The normal serum used was always from a species different to the ones used to raise the primary or secondary antibodies. Primary antibodies were then added at appropriate dilutions in PBS and the cells were incubated overnight in humidified atmosphere at 4°C. Negative control cell coverslips were also used to test the specificity of the staining, in which cells were incubated in blocking buffer and then in PBS in the absence of primary antibodies. Following incubation the cells were washed 3 times in PBS to remove traces of the primary antibodies and appropriately diluted secondary antibodies were added to each coverslip for 2 hours. To stain the nuclei, after 3 more washes in PBS the cells were incubated for 1 minute in 1:1000 DAPI (4'-6-Diamidino-2-phenylindole) in PBS solution. DAPI binds to natural double-stranded DNA forming blue fluorescent complexes with excitation wavelength at 345 nm and emission wavelength at 455 nm (Lin & Alfi, 1976). After a final wash in PBS each coverslip was rinsed in distilled water, blotted on tissue and mounted on a glass slide using Vectashield mountant. The coverslips were carefully sealed on the slides using nail varnish and then viewed under the microscope or stored at -20°C.

The cells were viewed under a fluorescence microscope (Zeiss Axioskop 2, Oberkochen, Germany) using the appropriate filter for each fluorochrome. Cells were viewed at excitation 343 nm with emission 480 nm for DAPI, at excitation 495 nm with emission 517 for FITC conjugated antibodies, and at excitation 581 nm with

emission 596 nm for TRITC conjugated antibodies. Images were captured using a Zeiss Axiocam HRc camera. The lenses used were a 20X Plan-NEOFLUAR, a 40X Plan-NEOFLUAR or a 100X EC Plan-NEOFLUAR. All primary and secondary antibodies used are listed in Table 2.6.1.

	<b>Specificity</b>	<b>Dilution</b>
<b>Primary antibodies</b>		
<b>Mouse anti rat anti-CD11b (OX-42)</b> Serotec MCA275EL	Recognises the CR3 receptor of ramified microglia.	1:500
<b>Mouse anti rat anti-ED1</b> Serotec MCA341	Recognises a glycoprotein on the lysosomal membranes of microglia, activation upregulates it.	1:100
<b>Rabbit polyclonal anti-MBP</b> Chemicon AB980	Binds to myelin basic protein. Mature oligodendrocyte marker.	1:200
<b>Mouse polyclonal anti-MOG</b> Sera-Lab NCI-031	Binds to myelin oligodendrocyte glycoprotein. Mature oligodendrocyte marker.	1:100
<b>Rabbit polyclonal anti-NG2</b> Santa Cruz Sc-20162	Binds to NG2, a chondroitin sulfate proteoglycan, expressed on the cell surface of OPCs.	1:50
<b>Rabbit anti mouse anti-iNOS</b> BD Transduction Lab 610333	Binds to inducible nitric oxide synthase, a marker of activated microglial cells.	1:250
<b>Goat anti-GFAP (N-18)</b> Santa Cruz Sc-6171	GFAP (Glial fibrillary acidic protein) is a specific astrocyte marker.	1:300
<b>Secondary Antibodies</b>		
<b>Goat anti-rabbit IgG TRITC</b> Sigma T6778	Binds to antibodies raised in rabbit.	1:500
<b>Rabbit anti-goat IgG FITC</b> <b>Sigma F9012</b>	Binds to antibodies raised in goat.	1:1000
<b>Sheep anti-mouse IgG FITC</b> Sigma F2883	Binds to antibodies raised in mouse.	1:100

**Table 2.6.1 Primary and secondary antibodies used for immunostaining**

## 2.7 Western blotting

Western blotting is an electrophoretic method used for separating proteins present in a biological sample and successfully identifying the presence of a specific protein in that sample (Burnette, 1981). Sodium dodecyl sulphate (SDS) gel electrophoresis (SDS-PAGE) employs polyacrylamide gels and buffers loaded with SDS to separate the proteins. Polypeptides are maintained in a denatured, negative charged state and are thus separated by their molecular weight. This is achieved by applying an electrical current, so that proteins covered in the negatively charged SDS move to the positively charged electrode through the acrylamide mesh of the gel and are separated according to size. This results in bigger protein molecules being retained by the polyacrylamide mesh, while the smaller (lighter) ones travel easier and move further down the gel. The proteins retained on the gel are then transferred onto a membrane where they are detected by using antibodies specific to the protein of interest. Western blotting was used to identify and semi-quantify cytosolic proteins that were produced after activation of primary microglia and BV-2 cells. Western blotting was also used to identify myelin basic protein (MBP) in oligodendroglial cultures.

### 2.7.1 Sample preparation

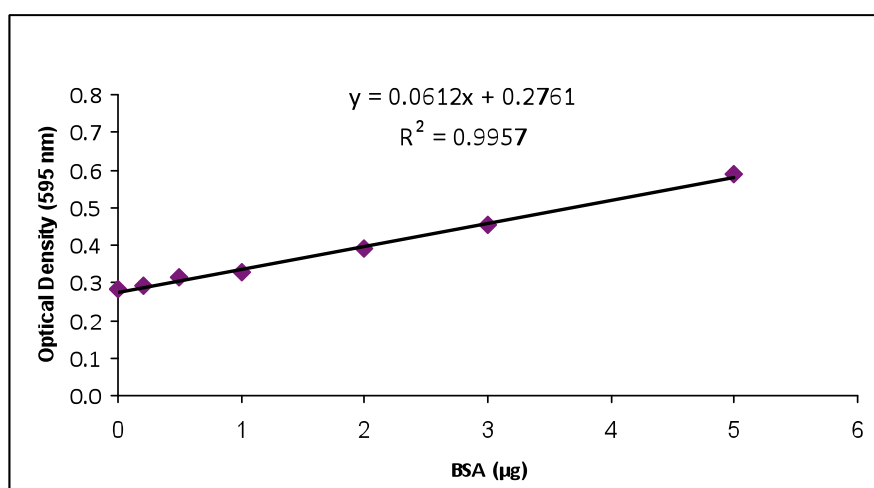
Primary microglia, BV-2 cells or oligodendrocytes (200,000 cells/well) were harvested into 30  $\mu$ l of lysis buffer. The formulation of the lysis buffer aimed at lysing the cells and solubilising the membranes as well as preserving the state of the proteins at the time of lysis (prevent phosphorylation, dephosphorylation and proteolysis). The lysis buffer consisted of 20 mM Tris-acetate buffer (pH 7), 1 mM ethylene diamine tetraacetic acid (EDTA) to chelate calcium, 1 mM ethylene glycol tetraacetic acid (EGTA) to chelate magnesium, 10 mM of the tyrosine phosphatase inhibitor sodium- $\beta$ -glycerophosphate, 1 mM of the tyrosine phosphatase sodium orthovanadate, 5% glycerol which depresses the solution's freezing temperature, 1% Triton X-100 membrane solubiliser, 0.27 M sucrose, 1 mM of the protease inhibitor benzamidine, 4  $\mu$ g/ml of the protease inhibitor leupeptin, 1  $\mu$ M of the phosphatase



inhibitor microcystin LR and 0.1% of the reducing agent  $\beta$ -mercaptoethanol (Evans & Pocock, 1999; Kingham & Pocock, 2000). Samples were left on ice for approximately 30 minutes and then stored at -20°C until used.

### 2.7.2 Bradford protein assay

The protein concentration of the cell lysates was calculated using the Bradford assay (Bradford, 1976). All the proteins studied were cytosolic, so the samples were centrifuged at 15,800g for 5 minutes at room temperature and the supernatants containing the cytosolic proteins were collected (Evans & Pocock, 1999). The pellets contained nuclei and membrane fragments. Each cytosolic sample was measured for its protein content. Into a 96 well plate 1 ml of each sample was added in triplicate and 200  $\mu$ l Bradford Reagent were added into each well. After the samples were mixed on an orbital shaker, the absorbance shift of the coomassie dye towards blue by the binding of protein was measured on a Tecan Genius plate reader at 595 nm. The protein concentration of the samples was determined using a bovine serum albumin (BSA) standard curve of known concentrations of BSA diluted in distilled H<sub>2</sub>O against optical density at 595 nm, which was constructed on the day of the experiment.



**Figure 2.7.2.1 Representative BSA standard curve**

BSA standards were made up on the day of the experiment by serial dilution of a 0.5 mg/ml solution and underwent the same protocol as the samples under investigation. A standard curve was plotted and cell lysate protein concentrations were determined against the standard curve.

### 2.7.3 Pouring SDS PAGE gels

A Bio-Rad mini gel apparatus was used to pour fresh sodium dodecyl sulfate (SDS) polyacrylamide gels (SDS PAGE). Two glass plates were assembled together, separated by spacers and mounted onto a pouring stand. Resolver and stacker gel composition is listed in Table 2.7.3.1. N, N, N', N'-tetramethylethylenediamine (TEMED) and ammonium persulfate (APS), both required for acrylamide polymerisation and gel formation, were added last just before pouring. Depending on the molecular weight of the protein under investigation, the percentage of acrylamide in the resolver gel could be adjusted, with higher percentage of acrylamide used for better separation of lower molecular weight proteins. The resolver gels used here were 10% for iNOS detection (130 KDa) or 12% for all other proteins. Resolver gel was first poured between the plates using a Pasteur pipette to a level of a couple of mm below where the comb would lie. A layer of 100% ethanol was pipetted on the acrylamide and the gel was allowed to set for approximately 30 minutes. After removing the ethanol and washing a few times with water to remove any unpolymerised acrylamide the stacker gel was poured in the same way, right up to the top of the plates and the comb was applied to form the wells, around 2 mm above the resolver gel level. After approximately 40 minutes the gels were ready to use and the gel apparatus could be assembled.

Resolver Gel (12%)	Resolver Gel (10%)	Stacker Gel (4%)
4 ml	3.33 ml	1.33ml
30% polyacrylamide	30% polyacrylamide	30% polyacrylamide
2.5 ml resolver buffer (1.15 M Tris-HCl, 0.1% SDS, pH 8.8)	2.5 ml resolver buffer (1.15 M Tris-HCl, 0.1% SDS, pH 8.8)	2.5 ml stacker buffer (0.38 M Tris-HCl, 0.1% SDS, pH 6.8)
3.5 ml H <sub>2</sub> O	4.17 ml H <sub>2</sub> O	6.17 ml H <sub>2</sub> O
50 µl 10% APS (fresh)	50 µl 10% APS (fresh)	50 µl 10% APS (fresh)
5 µl TEMED	5 µl TEMED	5 µl TEMED

**Table 2.7.3.1 Composition of resolver and stacker gels.**

#### **2.7.4 SDS PAGE electrophoresis**

Lysate volumes equivalent to 45 µg of protein as determined by the Bradford assay were run through the SDS PAGE gels. Samples were diluted 1:1 in sample buffer which consisted of 2% SDS, 10% glycerol, 2.5% mercaptoethanol, 125 mM Tris/HCl (pH 6.8) and a few bromophenol blue crystals (Laemmli, 1970), and were boiled for 4 minutes to denature the proteins. After mixing, the samples were ready to add into the gel wells. The gels were placed into their holder and into a tank. The wells were filled with running buffer (125 mM Tris/HCl, 1 M glycine, 0.01% SDS) and the samples were carefully added into each well. Standards and molecular weight markers (Bio-Rad Precision Plus Protein standards #161-0373, Hertfordshire, UK, Magic Mark, Invitrogen, Paisley, UK) were also added into wells. The tank was then filled with running buffer and the gels were electrophoresed at 120-130V until the proteins were separated as observed by the coloured molecular weight marker running simultaneously with the samples.

After the proteins were separated, the gel was carefully removed from the glass plates and equilibrated for 20 minutes in cold transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, 0.01% SDS). While the gel was equilibrating, a piece of polyvinylidene fluoride (PVDF) membrane (Immobilon P) the same size as the gel was activated in ice cold methanol for 1 minute, followed by 5 minutes in distilled water and approximately 20 minutes in cold transfer buffer. The gel was then transferred to a cassette and placed on top of a fibre pad soaked in transfer buffer and a piece of 3MM paper sheet. The membrane was placed on top of the gel, ensuring no bubbles were trapped between them, and finally a piece of 3MM paper sheet and a filter pad were added on top of the membrane. The cassette was assembled and slotted into its holder and placed in a tank filled with gently stirring cold transfer buffer, next to an ice pack. The gel was left to transfer overnight (16-18 hours) at 22V or for 40 minutes at 60V for low molecular weight protein detection (10-30 KDa).

### 2.7.5 Immunoblotting

After transfer completion the PVDF membrane was removed from the transfer tank, placed on a shaking platform and washed for 10 minutes in Tween-20 Tris buffer saline (TTBS: 10 mM Tris HCl, 150 mM NaCl, 0.5% Tween-20, pH 7.4). Subsequently the membrane was blocked for non-specific binding into blocking buffer (5% non-fat dried milk in TTBS, unless otherwise specified) for at least one hour with shaking. The next step was to incubate the PVDF membrane with an appropriate primary antibody diluted in blocking buffer for the time specified (Table 2.7.5.1). After three washes in TTBS the membrane was incubated with an appropriate secondary antibody conjugated to horse radish peroxidase (HRP) diluted in blocking buffer, for 1 hour (Table 2.7.5.1). After three last washes in TTBS the proteins of interest were ready to be detected by enhanced chemiluminescence. The membrane was gently drained and placed in a tray with 1 ml of solution 1 and 1 ml of solution 2 of ECL (Amersham Biosciences) for 1 minute. Subsequently the membrane was drained between two sheets of 3MM paper sheet, wrapped in transparent wrap film (Saran) and fixed on an X-ray film cassette. In a darkroom the protein bands were exposed on photographic film (Amersham Hyperfilm) for an appropriate amount of time depending on the intensity of the staining, immersed in developing solution, fixing solution and finally thoroughly rinsed in tap water. After drying the films were viewed, scanned and the bands semi-quantified using Gel-Pro Imager 32 software. Western blotting experiments were repeated in triplicate unless otherwise stated, so blots presented in this study are representative of three experiments.

The blots could be reprobed for a different protein of interest. For this purpose, the blots were washed in TTBS for 5 minutes and incubated in 1X Re-Blot Plus antibody stripping solution (Chemicon, Watford, UK) for 3 minutes. After a 5 minute wash in TTBS the blot was immunoblotted following the protocol from the non-specific blocking step. The blots were routinely reprobed with a  $\beta$ -actin antibody (Kingham & Pocock, 2000) to verify equal protein loading, since  $\beta$ -actin is a cytoskeleton protein abundant in all cell types.

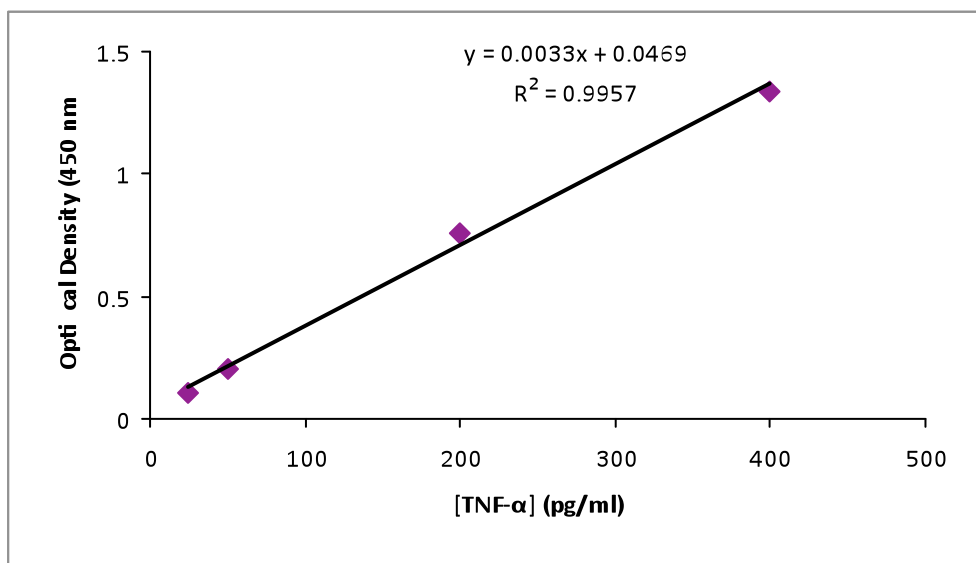
	Specifications	Dilution
<b>Primary antibodies</b>		
<b>Rabbit anti mouse anti-iNOS</b> BD Transduction Lab 610333	2 hours in 5% blocking buffer	1:5000
<b>Rabbit polyclonal anti-MBP</b> Chemicon AB980	Overnight at 4°C in 5% blocking buffer	1:2000
<b>Goat polyclonal anti-COX-2</b> Santa Cruz Sc-1746	Overnight at 4°C in 5% blocking buffer	1:750
<b>Goat polyclonal anti-GFAP (N-18)</b> Santa Cruz Sc-6171	Overnight at 4°C in 5% blocking buffer	1:500
<b>Mouse IgG1 anti-b-actin</b> Sigma #A5441	2 hours in 5% blocking buffer	1:2000
<b>Secondary Antibodies</b>		
<b>Goat <u>anti-rabbit</u> IgG-HRP</b> Santa Cruz Sc-2004	1 hour in 5% blocking buffer	1:1000
<b>Donkey <u>anti-goat</u> IgG-HRP</b> Santa Cruz Sc-2056	1 hour in 5% blocking buffer	1:1000
<b>Donkey <u>anti-mouse</u> IgG-HRP</b> Affinity BioReagents #SA1-100	1 hour in 5% blocking buffer	1:2000

**Table 2.7.5.1 Antibodies used for immunoblotting**

## 2.8 Enzyme-linked immunosorbent assay for TNF- $\alpha$ measurement

The enzyme-linked immunosorbent assay (ELISA) is a method that allows for the quantification of a protein in a given sample (Engvall & Perlmann, 1971; Van Weemen & Schuurs, 1971). A quantitative sandwich ELISA kit for the measurement of TNF- $\alpha$  (Quantikine Rat TNF- $\alpha$ , R&D Systems, Oxford, UK) was utilised to measure the quantity of TNF- $\alpha$  released in control and treated cell culture medium samples (Xaus et al., 2000). Sandwich ELISA is a variant of the ELISA technique where a primary monoclonal antibody is bound to a surface and then the antigen under investigation directly binds onto the bound antigen (Belanger et al., 1973). This is different from classic ELISA, where the sample under investigation directly binds to the surface. The advantage of sandwich ELISA is that the non-specific binding of the sample onto the surface is diminished. The antigen is then traced by a second, usually polyclonal antibody that binds to it.

The assay was performed following the manufacturer's instructions. 96 well plates precoated with a monoclonal rat anti-TNF- $\alpha$  specific antibody were used. 50  $\mu$ l of assay diluent were added into each well, followed by 50  $\mu$ l of standard rat TNF- $\alpha$  samples of known concentration, TNF- $\alpha$  kit control or medium samples. The samples were incubated for 2 hours at room temperature and were then washed four times with wash buffer. 100  $\mu$ l of rat anti-TNF- $\alpha$  polyclonal antibody conjugated to horseradish peroxidase (TNF- $\alpha$  conjugate) were then added for a 2 hour incubation at room temperature. After four washes with wash buffer 100  $\mu$ l of substrate solution were added to the wells for 30 minutes in the dark (colour reaction). After 30 minutes 100  $\mu$ l of hydrochloric acid solution (stop solution) were added to terminate the reaction, the wells were mixed and optical density was measured with a microplate reader (Anthos htII, Salzburg, Austria) set to 450 nm with the reference filter set to 540 nm to correct for optical imperfections. Optical density had to be measured within 30 minutes after the reaction termination. The concentration of TNF- $\alpha$  in the cell medium samples was determined against a standard curve plotted on the day of the experiment from the standard TNF- $\alpha$  samples used on the same 96 well plate. An example of a standard curve is shown in figure 2.8.1.



**Figure 2.8.1 Representative TNF- $\alpha$  standard curve**

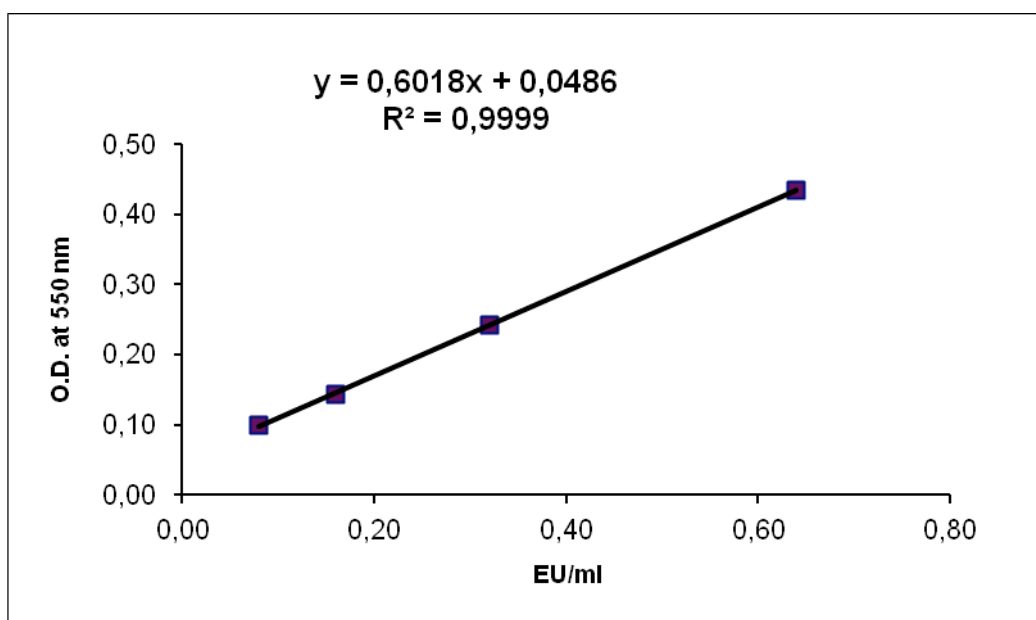
TNF- $\alpha$  standards were made up on the day of the experiment by serial dilution and underwent the same protocol as the samples under investigation. A standard curve was plotted and cell medium sample TNF- $\alpha$  concentrations were determined against the standard curve.

## 2.9 Endotoxin quantification by the Limulus Amebocyte Lysate assay

Following concerns over the purity of commercially available plasma-derived proteins used to activate microglia and the possibility that they might be contaminated with LPS (Weinstein et al., 2008), an endotoxin quantification kit (Limulus Amebocyte Lysate assay, LAL Pyrochrome, Associates of Cape Cod Incorporated) was used to test the proteins used to activate or treat microglia. LAL reagents used to detect endotoxin are prepared from an extract (lysate) from the amebocytes of the horseshoe crab, *Limulus polyphemus*. It was found that clotting in the LAL was triggered by endotoxin (Levin & Bang, 1968). The Pyrochrome assay contains the proteins of the clotting mechanism and allows for highly sensitive tests for endotoxin. The reaction consists of a cascade of enzyme activation steps terminating in the cleavage of the peptide coagulogen and the formation of insoluble coagulin, which forms a gel-clot. This interaction forms the basis of the assay. Endotoxin-activated LAL can also cleave small chromogenic peptides that result in the release of p-nitroaniline (pNA), which is yellow and absorbs light at 405 nm (Nakamura et al., 1977). Thus, when endotoxin is added to chromogenic LAL, color is formed and endotoxin can be quantified.

The method used was an endpoint chromogenic method that measured the amount of pNA produced following a fixed incubation period. Following the manufacturer's instructions, Pyrochrome was reconstituted in Pyrochrome buffer and had to be used within 8 hours. The samples were boiled, Pyrochrome was added at a 1:1 ratio (50  $\mu$ l : 50  $\mu$ l) and the samples were mixed for 30 seconds. The samples were incubated for a precise time (indicated by the Pyrochrome instructions for each batch) in a 37°C incubator. The reaction was terminated with 25  $\mu$ l 50% acetic acid and the optical density of the samples was measured at 405 nm. Together with the samples, a standard curve was constructed by serial dilutions of 50 endotoxin units/ml (EU/ml) control standard endotoxin (CSE). A positive control (a sample spiked with 0.5 EU/ml CSE) and a negative control (endotoxin free water) were also measured. The quantity of endotoxin in each sample was determined against the plotted standard curve. A representative standard curve is depicted in Figure 2.9.1.





**Figure 2.9.1 Representative control endotoxin standard curve**

50 endotoxin units/ml (EU/ml) control standard endotoxin (CSE) was serially diluted on the day of the experiment and the CSE standards underwent the same protocol as the samples under investigation. A standard curve was plotted and endotoxin contamination of the samples was determined against the standard curve.

## 2.10 Statistical analysis

All experiments were performed in triplicate unless otherwise stated. In microscopy experiments, cells were counted on three coverslips (4 fields of view counted per coverslip) per treatment from three independent experiments. Data were expressed as a percentage of the total number of cells counted per field. Therefore the experimental data presented in this study were subjected to statistical analysis to compare treatments to control groups. To compare a single treatment with a control group, two tailed t-tests were performed. To compare many treatments to a common control group or to other treatments, one way analysis of variance (ANOVA) was used in combination with the Tukey post hoc test. *P* values <0.05 were considered statistically significant. Where indicated, *p* values were ns (not statistically significant) >0.05, \*<0.05, \*\*<0.01 and \*\*\*<0.001.

### **3. OPTIMISATION OF PRIMARY GLIAL CULTURES**

### 3.1 Introduction

The different cell populations of the central nervous system have distinct functions and characteristics (see 1.1). Their connections and communication contribute towards tissue homeostasis and function, but when studying the unique properties of each cell population and the interactions between them, it is essential to culture them separately. Establishing reliable methods to isolate and maintain purified populations of cells is a powerful tool in neuroscience.

*In vitro* culturing of homogenous cell populations is demanding since isolated cells have far less trophic support than they do *in vivo*. Mixed glial cultures are easy to establish and viable for long periods of time (McCarthy & de Vellis, 1980; Floden & Combs, 2007), but when attempting to individually culture cell populations, the demands are greater and the cell viability falls rapidly with cell population purity. Astrocytes *in vitro* support other glial populations and neurones. For example astrocytes in neuronal cultures buffer glutamate and thus make neurones resistant to high concentrations of external glutamate (Rosenberg & Aizenman, 1989). Trophic factor secretion from astrocytes supports motor neurone survival (Albrecht et al. 2002) and promotes stem cell differentiation into neurones (Song et al., 2002). Oligodendrocyte death *in vitro* is attenuated in the presence of astrocytes that interact with oligodendrocyte membrane integrins (Corley et al., 2001).

To study the interactions between microglial cells and oligodendrocytes, a cell culture protocol was optimised, based on the widely used shaking method for the culture of rat glial cells (McCarthy & de Vellis, 1980). With this protocol, pure cortical microglial and oligodendrocyte cultures from a primary mixed glial culture derived from 5-6 day old Sprague Dawley rats can be obtained. Mixed cultures of microglia and oligodendrocytes of different maturation stages can also be obtained. This method allowed for astrocytes to be cultured after the removal of microglia and oligodendrocytes. In the rat glial shaking method cortical neurones were not viable unless the pups were younger than two days of age (McCarthy & de Vellis, 1980). In newborn rat preparations neurones could survive and differentiate on a confluent astrocyte layer (Hildebrand et al., 1997).

## **3.2 Optimisation and characterisation of primary microglial cultures**

### **3.2.1 Introduction**

Microglia have been successfully isolated from rat brain using a method based on isolation of the microglial fraction at the interface of 35% and 70% percoll gradients (Havenith et al., 1998; Kingham & Pocock, 2000). This method results in highly pure microglial cells (Kingham & Pocock, 2000). Another widely used method for the isolation of microglia from rat brain is the method based on the culture of mixed glial cultures and the differential detachment of microglial cells that rest on the astrocyte layer using an enzymatic method for tissue dissociation (Raff et al., 1979; Giulian & Baker, 1986; Vincent et al., 1996). In this thesis the differential detaching method was selected and optimised, in an attempt to culture mixed cultures and deploy all three glial populations, thus reducing animal use.

### **3.2.2 Optimisation of the microglial culture method**

For culturing mixed glia and isolating the microglia, the cortical tissue isolated from 5 day old rat pups was mechanically dissociated. This strategy is used for oligodendrocyte cell isolation from mixed glia cultures (McCarthy & de Vellis, 1980), whilst for microglia, enzymatic dissociation of the tissue is more widely used (Raff et al., 1979). The microglia on the astrocyte layer were ready to be removed when the astrocyte layer was confluent and many small microglia could be seen. Microglia can be detached from 7 DIV (Giulian & Baker, 1986) until 16 DIV (Vincent et al., 1996). The longer the mixed cultures are maintained the more microglia develop. 11-12 DIV was the time selected to detach the microglia, because at that stage the astrocyte layer was 100% confluent and the OPCs in the culture flasks had not started differentiating into oligodendrocytes and were also ready to be shaken. Shaking at an orbital shaker at 125 rpm for 5 hours (Stuart SSM1, SLS, Nottingham, UK) resulted in a satisfactory yield of microglia with no significant contamination with OPCs or astrocytes. This shake was preferred over a more intense shake for less time, which has been reported to produce less homogenous cells (Giulian & Baker, 1986).

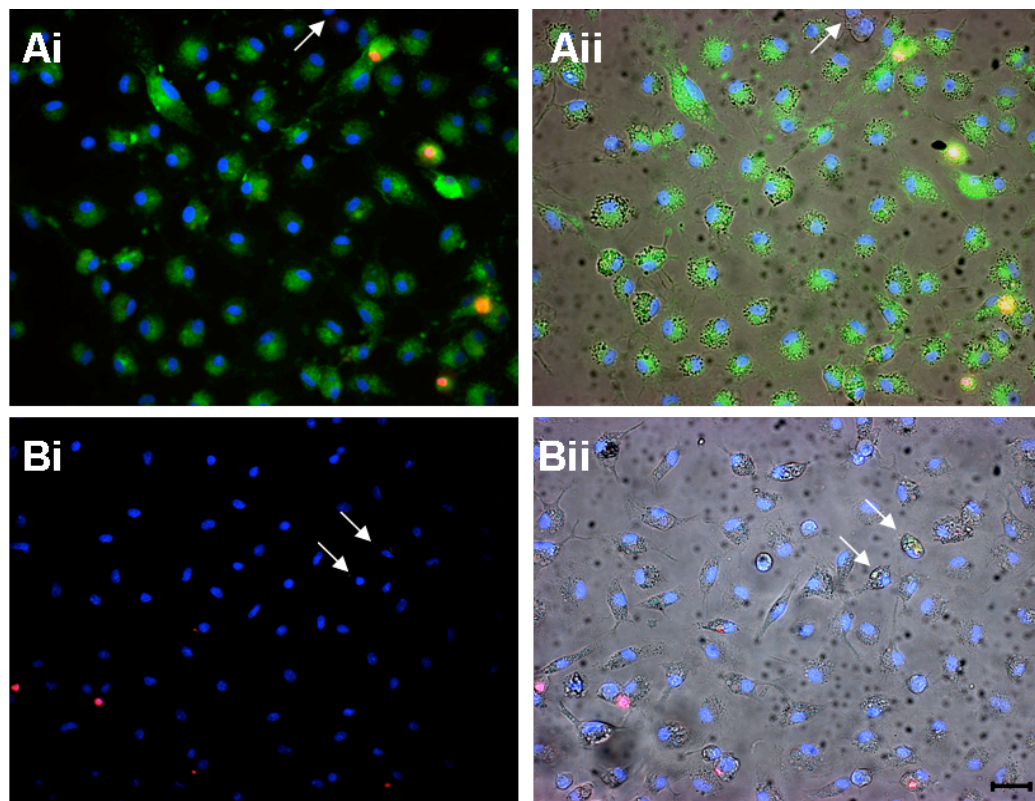
### 3.2.3 Purity of the microglial preparation

A line of immunofluorescence experiments was performed to assess the purity of microglia obtained from the shaking method. Live microglia plated onto uncoated glass coverslips were stained with FITC-conjugated *Bandeiraea simplicifolia* isolectin B<sub>4</sub> (BSI-B<sub>4</sub>). BSI-B<sub>4</sub> is a lectin with exclusive affinity for the  $\alpha$ -D-galactosyl oligosaccharide residue (Wood et al., 1979) and it selectively binds to microglia in the brain (Streit & Kreutzberg, 1987). Microglia labelled with BSI-B<sub>4</sub> were also stained with Hoechst 33342 (Hoechst) which is a nuclear stain and propidium iodide (PI) which is a DNA stain that is membrane impermeable and can only stain dead cells. The cells were viewed under a fluorescence microscope (Zeiss Axioskop 2, Oberkochen, Germany) using the appropriate filter for each fluorochrome. Cells were viewed at excitation 343 nm with emission 480 nm for Hoechst 33342 and at excitation 495 nm with emission 517 nm for FITC conjugated BSI-B<sub>4</sub>. Images were captured using a Zeiss AxioCam HRc camera. The lenses used were a 20X Plan-NEOFLUAR, a 40X Plan-NEOFLUAR or a 100X EC Plan-NEOFLUAR. The number of green fluorescent cells (FITC-BSI-B<sub>4</sub>) in each field was counted and the percentage to blue cells (Hoechst) was calculated. The experiment was repeated in triplicate and for each experiment at least three coverslips were viewed and at least four fields were counted per coverslip. The percentage of microglia was 96.5 %  $\pm$  0.5 (SEM) (n=3), with the contaminating cells being mostly cells of the oligodendrocyte lineage or astrocytes. Negative controls were also assessed by taking pictures of non stained coverslips through the FITC filter. The inherent fluorescent properties of microglial vesicular structures have been reported (Mayer et al., 2001), thus care should be taken not to mistake vesicular autofluorescence for immunolabelling. Representative images of BSI-B<sub>4</sub> stained microglia as well as negative controls are shown in Figure 3.2.3.1.

Microglia were also stained with the microglial specific markers OX-42 and ED1. OX-42 is a macrophage specific marker that recognises the CR3 complement receptor CD11b/CD18 in rat cells and is thus a specific marker for resting and activated microglia (Graeber et al., 1989). ED1 recognises a glycoprotein on the lysosomal membranes in rat macrophages and exhibits a punctuate staining pattern

(Dijkstra et al., 1985; Damoiseaux et al., 1994). High levels of ED1 are associated with activated microglia (Graeber et al., 1990) and the amount of ED1 expression in a single cell can be correlated to the cell's phagocytic activity (Damoiseaux et al., 1994). Representative images of primary rat microglia stained with ED1, OX-42 as well as the astrocyte specific marker GFAP can be seen in Figure 3.2.3.2.

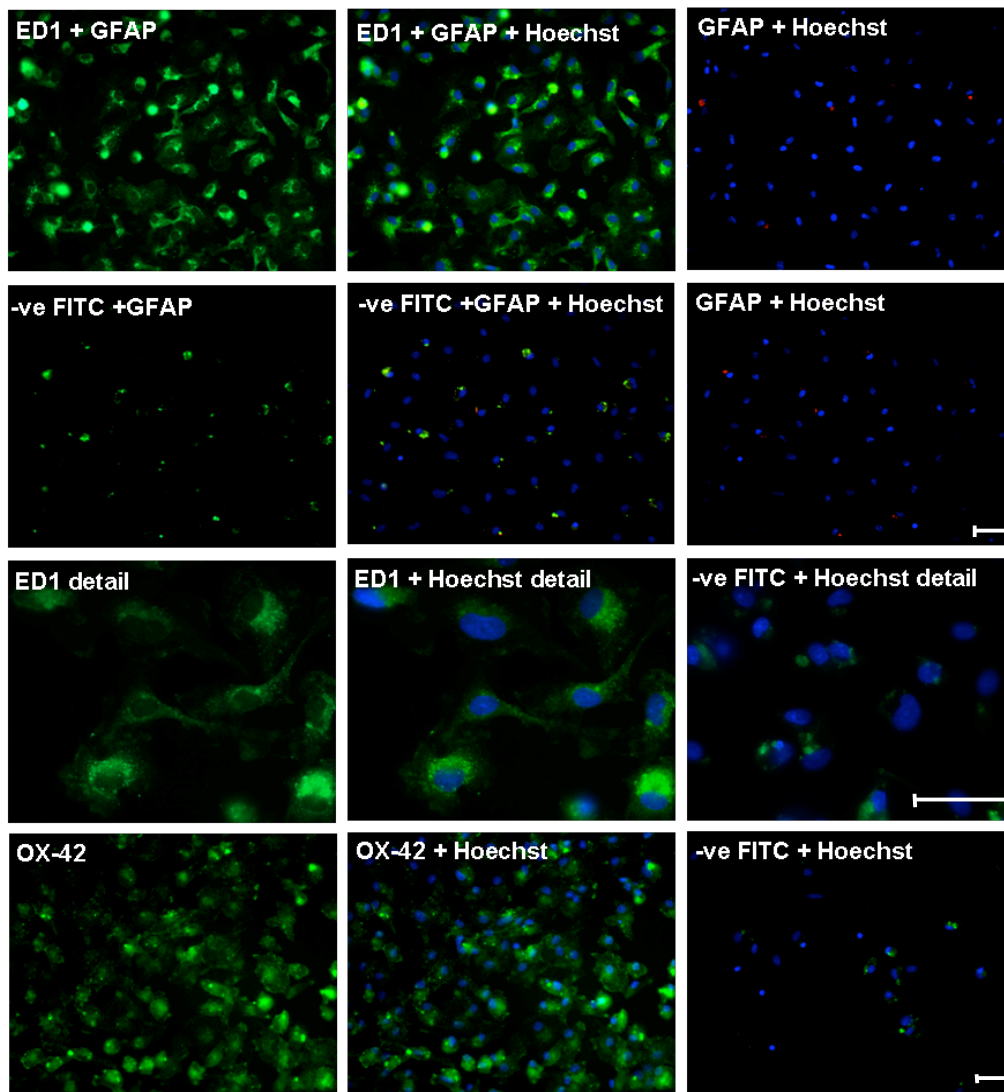
Finally, microglia, or the cells that remained on the flasks following complete removal of microglia by shaking, were lysed and homogenised. Western blotting was performed to assess whether these cell fractions expressed inducible nitric oxide synthase (iNOS) or GFAP. It was shown that microglia did not express the astrocytic marker GFAP, whilst GFAP was amply expressed in the astrocyte - oligodendrocyte lysates. iNOS was strongly expressed following 24 hour microglial activation with 0.1 µg/ml lipopolysaccharide (LPS). iNOS was not expressed after LPS activation of the astrocyte containing cultures (Figure 3.2.3.3).



**Figure 3.2.3.1 Microglial cell culture purity**

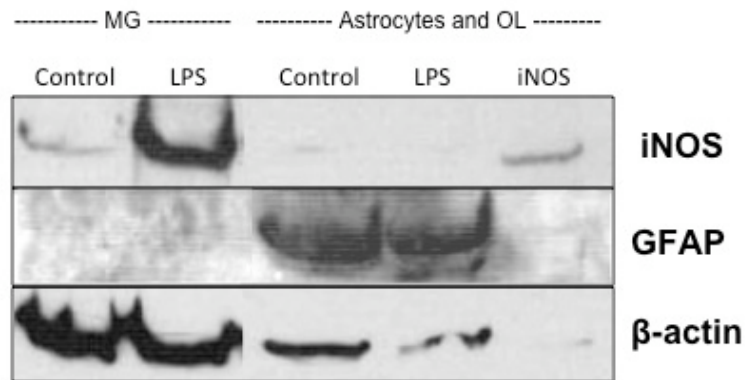
Live microglia plated on uncoated glass coverslips, stained with FITC-conjugated *Bandeiraea simplicifolia* isolectin B<sub>4</sub> (BSI-B<sub>4</sub>) for 2 hours. Cells were counter stained with the nuclear stains Hoechst (live cells) and propidium iodide (PI) (dead cells). (Ai) is a representative image of microglia stained for BSI-B<sub>4</sub>, Hoechst and PI. Microglia were 96.5%  $\pm$  0.5 (SEM) (n=3) pure, with the contaminating cells being astrocytes or cells of the oligodendrocyte lineage. A bright field phase contrast of the same picture (Aii) allows for cell details to be viewed. The arrow indicates two cells that did not stain with BSI-B<sub>4</sub> and most probably belong to the oligodendrocyte lineage. Negative controls were stained with Hoechst and PI and visualized through the FITC filter (Bi). Microglia exhibit intense vesicular structure autofluorescence (arrows). The autofluorescence is limited to the vesicular structures and is absent from cell bodies and processes, as can be seen with a bright field phase contrast picture (Bii). Scale bar = 20  $\mu$ m.





**Figure 3.2.3.2 Fluorescent staining on fixed primary microglia**

Microglia fixed on uncoated glass coverslips, stained with the specific microglial markers ED1 (green) or OX-42 (green). Some coverslips were also stained with the astrocyte specific marker GFAP (red), but no astrocytes were detected. Cells were counter stained with the nuclear stain DAPI (blue). The first column depicts images captured through the FITC channel. The second column shows nuclear staining (Hoechst) over-layed on the first column. The third column depicts negative control images captured through the FITC or TRITC channel without the use of primary and FITC conjugated antibodies. Microglia exhibit intense vesicular structure autofluorescence which can be seen in negative (–ve) FITC images where both the primary and the secondary antibodies have been omitted. The autofluorescence exhibited by microglia also leaks into the TRITC channel as can be seen if only the TRITC channel is captured (GFAP + Hoechst images). Scale bar = 20  $\mu$ m.



**Figure 3.2.3.3 Microglial cell cultures expressed iNOS but not GFAP**

Microglial or astrocyte - oligodendrocyte lysates were left untreated or were activated for 24 hours with 0.1 µg/ml LPS. The cells were lysed and western blotting for the expression of iNOS or GFAP in the lysates was performed. Western blotting was performed to assess whether these cell fractions expressed inducible nitric oxide synthase (iNOS) or GFAP. Microglia did not express the astrocytic marker GFAP, whilst GFAP was amply expressed in the astrocyte - oligodendrocyte lysates. iNOS was strongly expressed following 24 hour microglial activation with 0.1 µg/ml lipopolysaccharide (LPS). iNOS was not expressed after LPS activation of the astrocyte containing cultures. β-actin was used as a loading control.

### **3.2.4 Discussion**

It was shown here that highly pure microglia (96.5% +/- 0.5; SEM) can be isolated by controlled agitation of mixed glial cultures. Additionally it was shown that the microglial fraction predominantly expressed iNOS following activation (Figure 3.2.3.3; Saura, 2007).

With this method a number of microglia remained onto the astrocyte layer and could be removed with a second shake, but that was avoided to ensure cell preparation purity. If the microglia obtained were not highly homogenous, an additional step of washing the cells with PBS 15-20 minutes after plating could help remove any loosely adherent non-microglial cells. This was not considered necessary in the preparations used in this study.

### **3.3 Optimisation and characterisation of primary oligodendrocyte cultures**

#### **3.3.1 Introduction**

For the isolation of homogenous oligodendrocyte cell preparations from perinatal rat brain a number of different methods have been employed. The most popular method used involves the preparation of a mixed glial culture from which removal of the oligodendrocyte precursors is achieved by vigorously shaking the mixed glial culture (McCarthy & de Vellis, 1980). In this thesis this method was modified for the isolation of oligodendrocyte cultures from 5 day old rats. Other methods for the isolation of oligodendrocytes include the use of a Percoll centrifugation gradient for the separation of OPCs (86% pure; Lubetzki et al., 1991), or the growth of OPCs as aggregates (oligospheres) with 98% of the cells staining for an OPC marker (Avellana-Adalid et al., 1996). Another method that ensures cell preparation purity is the immunopanning method, which uses mixed glial cultures as a starting point and oligodendrocyte lineage specific antibody coated dishes to bind the cells desired (Collarini, 1996). OPCs have also been isolated from rat optic nerves (Raff et al., 1983; Tang et al., 2000).

#### **3.3.2 Optimisation of the oligodendrocyte culture method**

Most of the commonly used methods for the isolation of homogenous oligodendrocyte cell preparations are based on the isolation of OPC cells that can then develop into mature oligodendrocytes *in vitro*. The maturation of OPCs *in vitro* is a demanding process, since OPCs cultured in medium containing more than 5% serum develop into type 2 astrocytes, cells that express both astrocyte and oligodendrocyte markers, whilst they acquire a stellate morphology (Raff et al., 1983). Thus, different defined medium formulations have been used to provide developing OPCs with necessary support to survive and differentiate into oligodendrocytes in serum free conditions (Bottenstein & Sato, 1979; Raff et al., 1983; Louis et al., 1992). The media formulations used in this study (Armstrong, 1998; Li et al., 2005) did promote OPC differentiation into oligodendrocytes, but by

day 6 *in vitro*, when cells had successfully differentiated and MBP was amply detected, around 40% of the cells had died. To promote cell survival the media formulations tried were combined (oligodendrocyte medium, Table 3.3.2.1) and cell death at 6 DIV was reduced to approximately 23% (Figure 3.3.2.1). Cell survival could also be significantly enhanced when the serum percentage in the medium was increased to 2% after the OPCs had entered the differentiation process, although that was avoided or done as late as possible since even this low percentage of serum allowed some cells to differentiate into type 2 astrocytes.

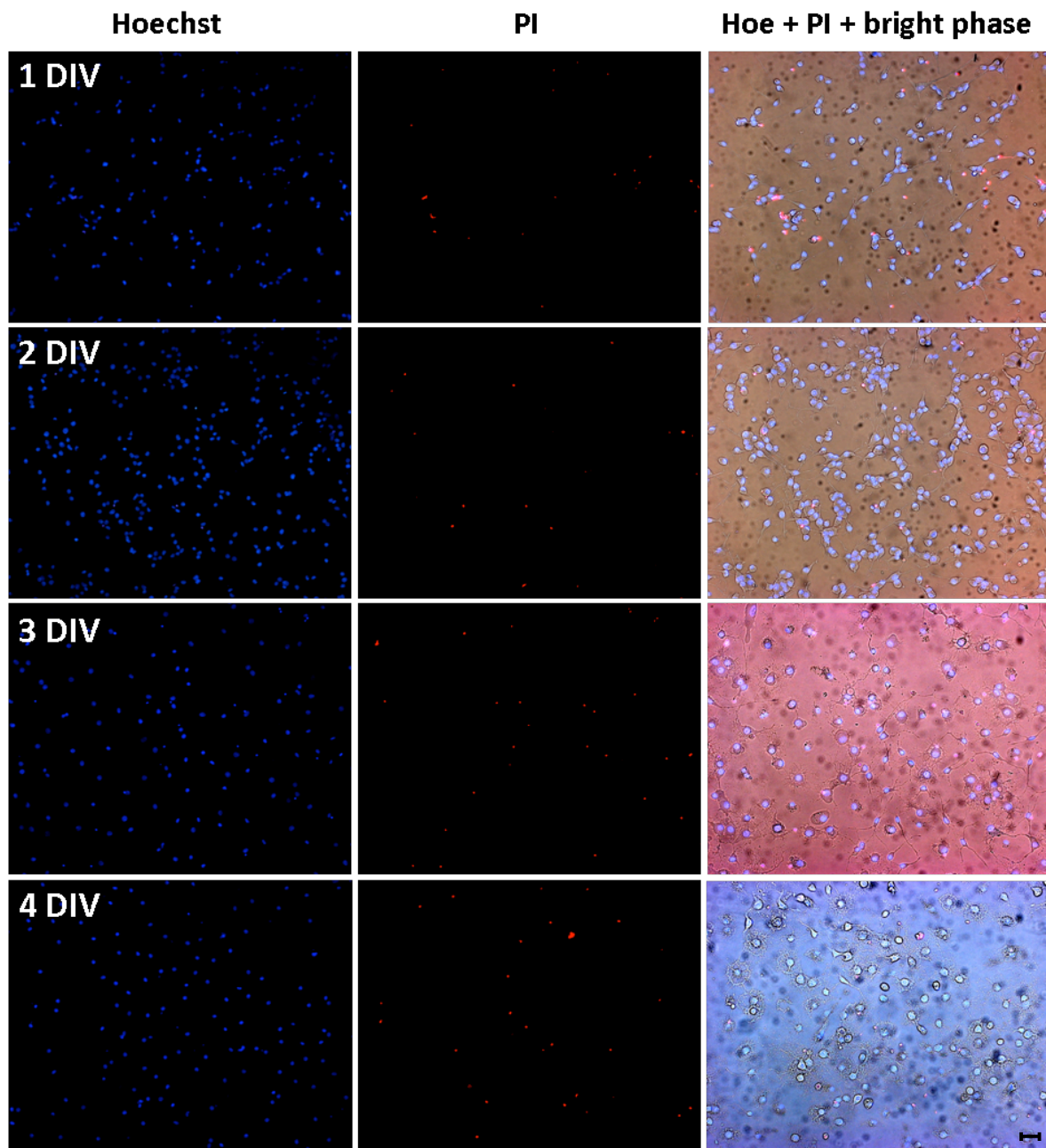
One more modification to the method was the use of 5 day old rats rather than 2 day old. The cell survival and morphology remained the same in both cases whilst 5 day old pups produced a higher yield of cells.

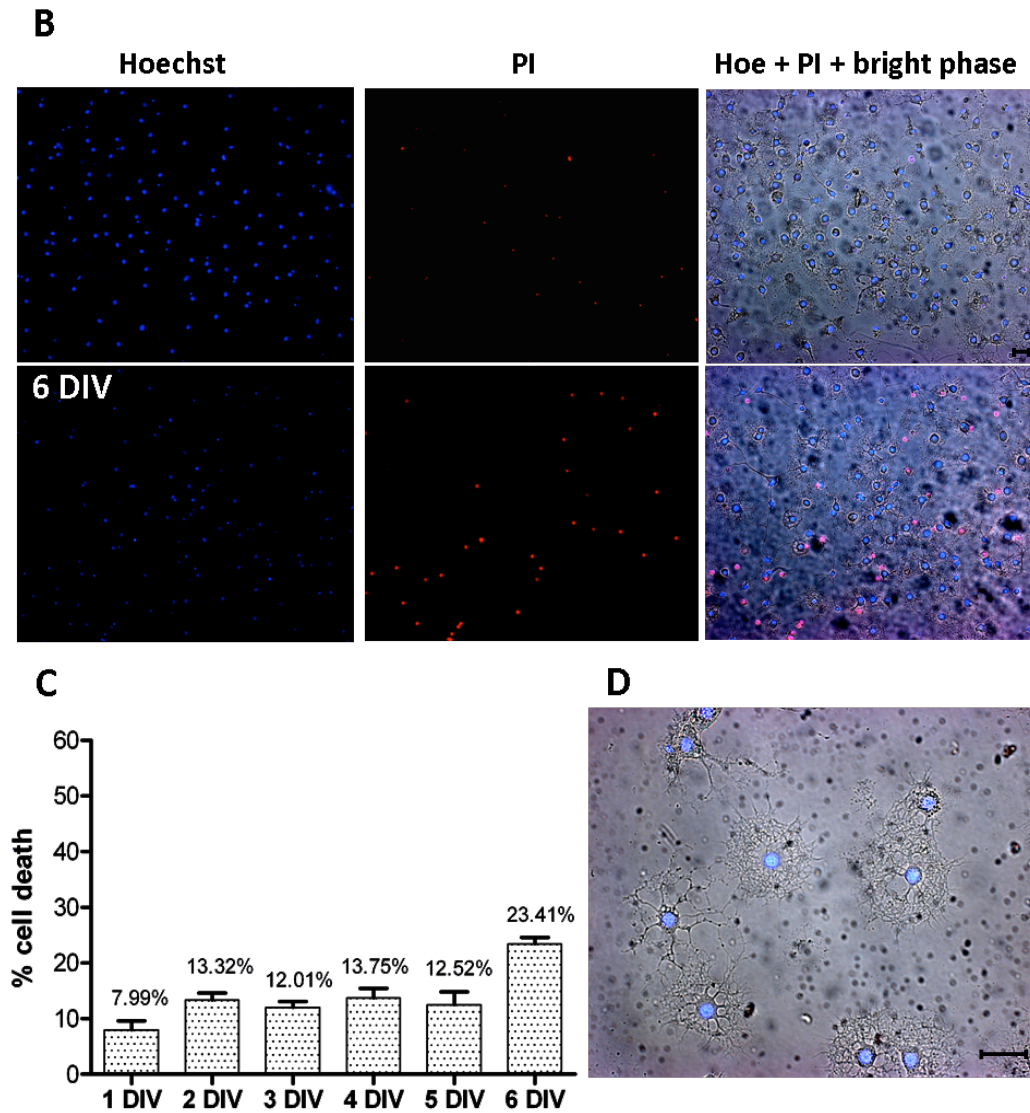
Oligodendrocyte medium	Armstrong 1998	Li et al. 2008
DMEM (with NaHCO <sub>3</sub> )	+	+
BSA 0.1%	-	+
Sodium pyruvate 1mM	+	-
Insulin 5 µg/ml	-	+ (50 µg/ml)
Transferrin 50 µg/ml	+	+
Selenium 30 nM	+	+
Tri-iodothyronine (T3)400 µg/ml	+	-
Biotin 5 ng/ml	-	+
Hydrocortisone 10 nM	-	+
FBS 0.5%	+	-
Gentamycin 25 µg/ml	+	-
Penicillin 100 U/ml	-	-
Streptomycin 100 mg/ml	-	-

**Table 3.3.2.1 Comparison of different oligodendrocyte media**

Comparison of the oligodendrocyte medium formulated to promote OPC survival and differentiation into oligodendrocytes (oligodendrocyte medium) with the media formulations initially used and combined (Armstrong, 1998, Li et al., 2008).

**A**





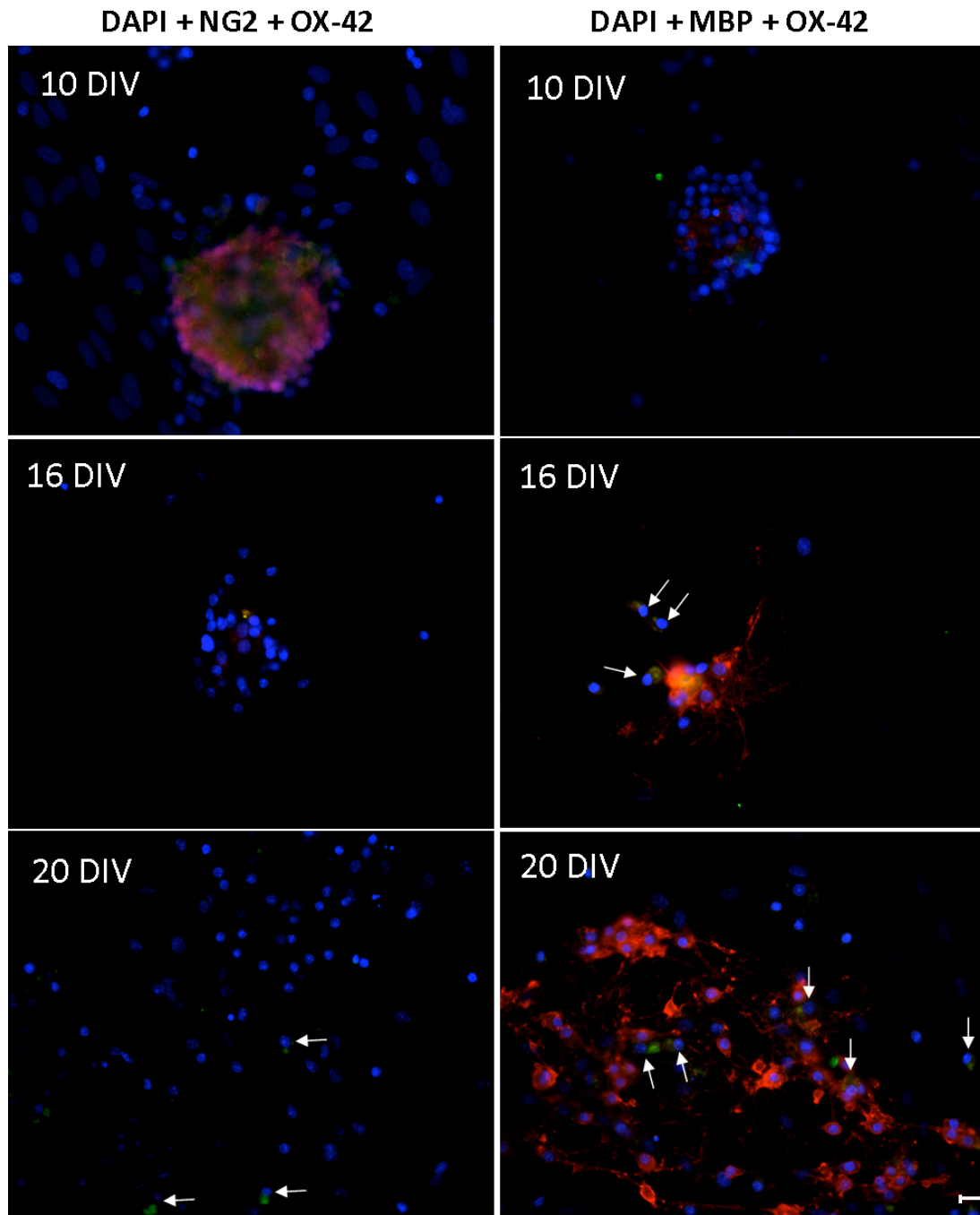
**Figure 3.3.2.1 Survival of maturing oligodendrocyte cell cultures**

Death levels of maturing OPCs *in vitro* were assessed by staining the cells with Hoechst (blue) and PI (red). Hoechst and PI images alone or with bright field from each day *in vitro* (A) (1-4) DIV and (B) (5-6) DIV are shown. 3 coverslips were assessed per condition and at least 3 pictures were taken per coverslip. The percentage of dead cells (PI) was counted in each field. At 6 DIV 23.41% of the cells stained for PI (C). At 6 DIV the cells had ample processes and resemble typical oligodendrocytes by morphological observation (D). This experiment was repeated in duplicate. Scale bar = 20  $\mu$ m.

### 3.3.3 Maturation and characterisation of *in vitro* oligodendrocytes

The maturation of oligodendrocytes *in vitro* was studied in mixed glial cultures plated onto PDL coated glass coverslips. After a week in culture a confluent monolayer of astrocytes had formed, on which the microglia and OPCs developed. At 9-12 DIV clumps of OPCs could be viewed on the astrocyte layer (Figure 3.3.2.2). These cells stained for the OPC marker NG2. NG2 is a chondroitin sulfate proteoglycan, which is expressed on the cell surface of immature progenitor cells such as OPCs (Stallcup & Beasley, 1987; Levine et al., 1993). 12 DIV was the time selected to shake the mixed glial cultures and obtain pure microglia and oligodendrocytes. At 16 DIV (time point that would correspond to 4 DIV pure oligodendrocyte cultures), OPCs were no longer present, having differentiated into process bearing oligodendrocytes that could be stained with the oligodendrocyte marker myelin basic protein (MBP; Roussel et al., 1983). MBP is the most abundant constituent protein of myelin and is present in differentiated, myelinating oligodendrocytes (Figure 3.3.2.2). At 20 DIV (time point that would correspond to 8 DIV pure oligodendrocyte cultures), the differentiated oligodendrocytes had developed an extensive network of myelinating processes. No OPCs were present (Figure 3.3.3.1).





**Figure 3.3.3.1 Maturation of oligodendrocytes *in vitro***

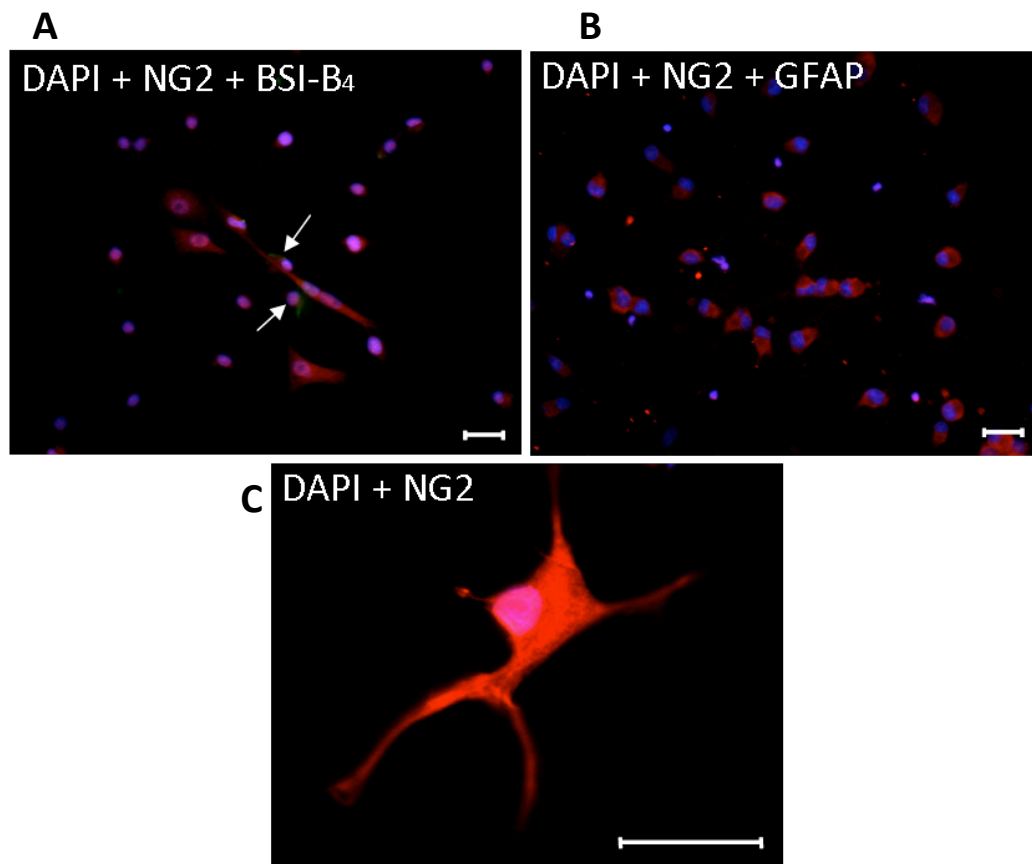
The maturation of OPCs to oligodendrocytes was studied by immunostaining fixed coverslips of mixed glia at different time points. Cells were stained with OX-42 (green) for microglia and DAPI (blue) to stain all nuclei, and with the OPC marker NG2 (red, left column) or the oligodendrocyte marker MBP (red, right column). Microglia are indicated with arrows. At 10 DIV clumps of OPCs formed on top of the astrocyte layer (unstained cells). At 16 DIV oligodendrocytes developed from the clumps, with fine, MBP staining processes. At 20 DIV oligodendrocytes formed an extended network of myelinating processes. No OPCs were detected after 16 DIV. Scale bar = 20  $\mu$ m.

### **3.3.4 Purity of the oligodendrocyte preparation**

To assess the purity of the oligodendrocytes obtained by using the shaking method a number of immunofluorescence experiments was performed. Both the OPC and the 6 DIV oligodendrocyte culture purity were assessed, even though the latter resulted from OPC maturation. Both culture time point preparation purities were assessed to ensure that contaminating cells did not proliferate during the maturation period and that OPCs only differentiated into oligodendrocytes. Fixed OPC or oligodendrocyte cultures plated onto PDL coated glass coverslips were stained with their specific markers NG2 and MBP as well as the microglial specific marker FITC-conjugated BSI-B<sub>4</sub> or the astroglial marker GFAP. Nuclei were stained with the membrane permeable DNA binding stain DAPI. The cells were viewed under the microscope and pictures were taken.

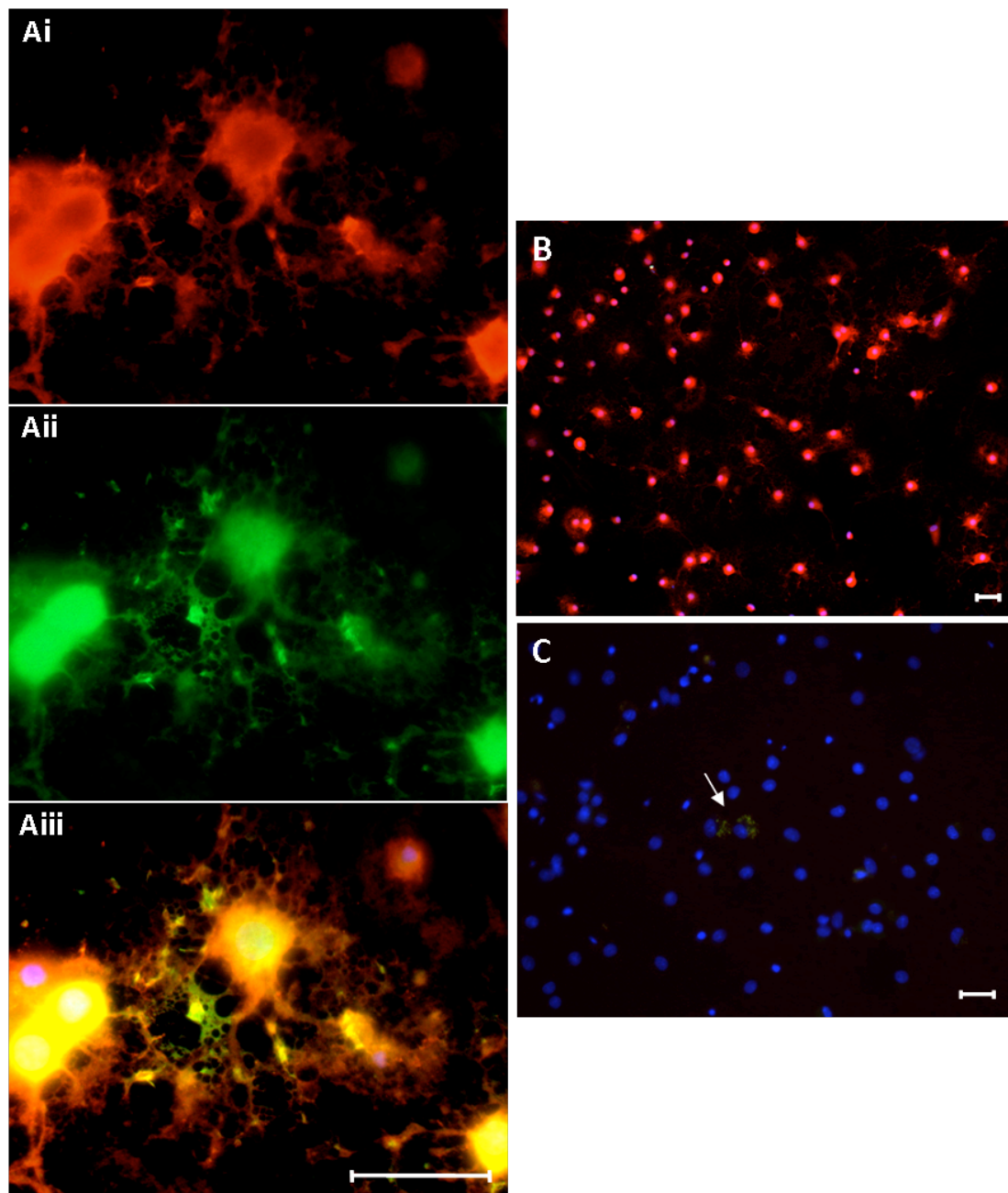
To calculate the purity of the OPC preparation the number of red fluorescent cells (NG2-positive) in each field was counted and the percentage to total number of cells was calculated. The experiment was repeated in triplicate and for each experiment at least three coverslips were viewed and at least four fields were counted per coverslip. The percentage of OPCs was 93.8 %  $\pm$  1.3 (SEM) (n=3), with the contaminating cells being predominantly microglia. Representative images of OPCs in culture are shown in Figure 3.3.4.1.

To calculate the preparation purity of 6 DIV oligodendrocytes the number of red fluorescent cells (MBP) in each field was counted and the percentage to total number of cells was calculated. The experiment was repeated in quadruplicate and for each experiment at least three coverslips were viewed and at least four fields were counted per coverslip. The percentage of 6 DIV oligodendrocytes was 91.9 %  $\pm$  0.7 (SEM), with the contaminating cells being predominantly microglia and less frequently astrocytes or fibroblasts. Representative images of oligodendrocytes in culture stained with MBP as well as the constitutive myelin protein myelin oligodendrocyte glycoprotein (MOG) are shown in Figures 3.3.4.2 and 3.3.4.3.



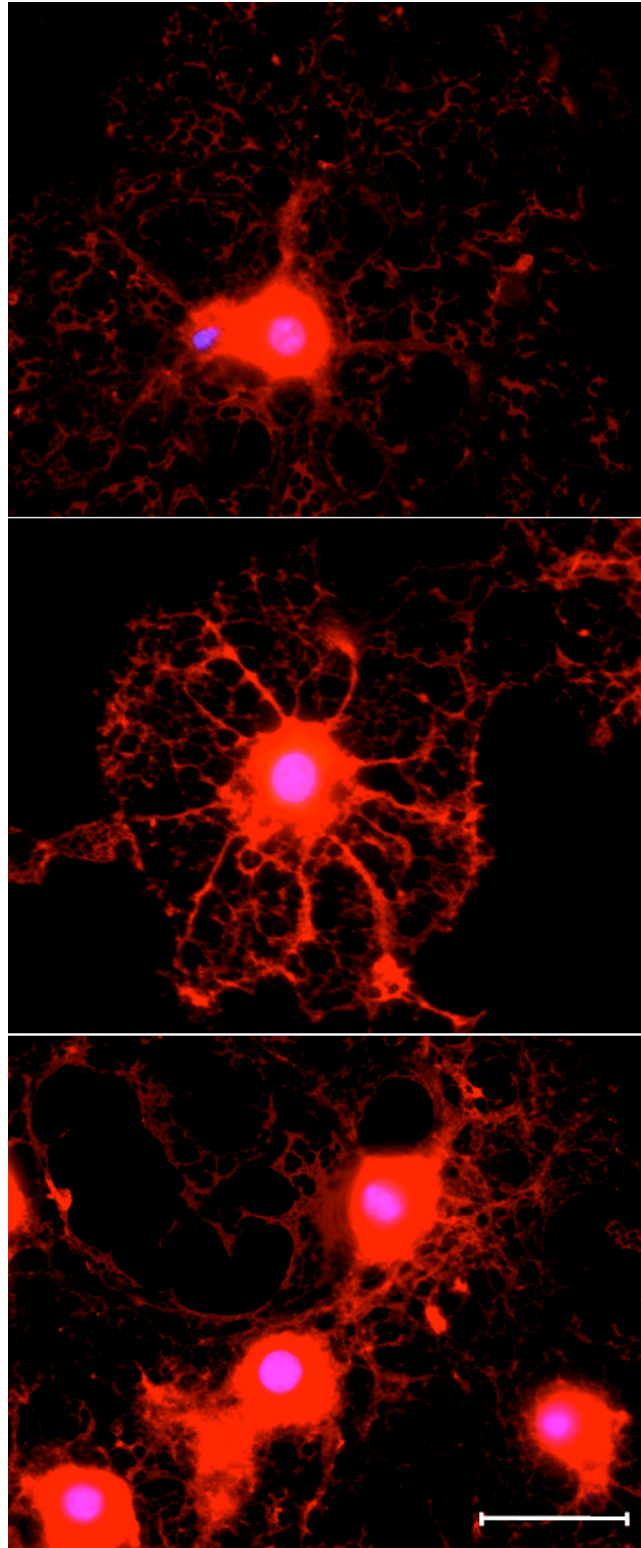
**Figure 3.3.4.1 Oligodendrocyte precursor cell (OPC) culture purity**

Representative images of OPCs plated on PDL coated glass coverslips, stained with NG2 (red), FITC-conjugated BSI-B<sub>4</sub> (green), GFAP (green) and the nuclear stain DAPI (blue). (A) is a representative image of OPCs stained with NG2 and BSI-B<sub>4</sub>. The arrows indicate two contaminating microglia. (B) is a representative image of OPCs stained with NG2 and GFAP. No astrocytes were detected. OPCs were 92.8%  $\pm$  1.3 (SEM) (n=3) pure, with the contaminating cells being predominantly microglia. (C) is a late precursor cell seen in detail (x 100 magnification). Scale bar = 20  $\mu$ m.



**Figure 3.3.4.2 6 DIV mature oligodendrocyte culture purity**

Representative images of 6 DIV oligodendrocytes plated on PDL coated glass coverslips, stained with MBP (red), MOG (green) or FITC-conjugated BSI-B<sub>4</sub> (green), and the nuclear stain DAPI (blue). (A) is a x 100 magnification representative image of oligodendrocytes stained with the two myelin markers MBP (i) and MOG (ii). The images were also merged (iii). (B) is a representative field of oligodendrocytes used to count cells and assess culture purity, which was calculated to be 91.9%  $\pm$  0.7 (SEM). In (C) the arrow indicates two contaminating microglia stained with BSI-B<sub>4</sub>. Scale bar = 20  $\mu$ m.



**Figure 3.3.4.3 6 DIV mature oligodendrocytes**

Representative x 100 magnification images of 6 DIV oligodendrocytes plated on PDL coated glass coverslips, stained with MBP (red), and the nuclear stain DAPI (blue). Scale bar = 20  $\mu\text{m}$ .

### 3.3.5 Discussion

We show here that pure OPCs (**93.8% +/- 1.3; SEM**) and mature 6 DIV oligodendrocytes (**91.9 +/- 0.7; SEM**) can be obtained by controlled agitation of mixed glial cultures (Table 3.5.1 A). The slightly lower percentage of mature oligodendrocytes in culture most possibly reflects the increase in oligodendrocyte death after 6 DIV, with contaminating microglia or astrocyte numbers not being affected. The physiological properties of oligodendrocyte lineage cells change with maturation, it is thus important to distinguish between the different maturation stages when studying the cell properties *in vitro*. Oligodendrocyte preparations were routinely assessed for cell purity and if the percentage of oligodendrocytes fell below 90% the cells were not used.

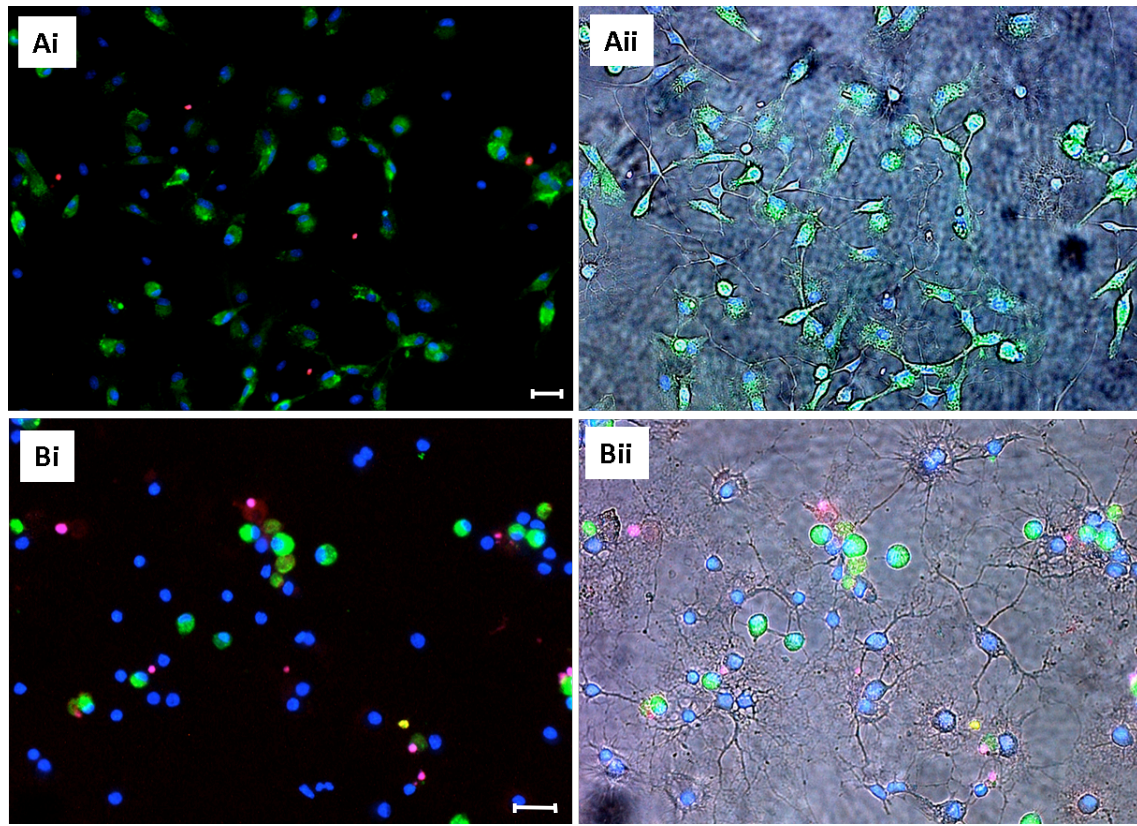
### 3.4 Optimisation and characterisation of microglial - oligodendrocyte co-cultures

To study interactions between microglia and oligodendrocytes, the two cell populations were cultured together. After the initial shake for removal of pure microglia, the mixed glial cultures (with many microglia remaining on the astrocyte layer) were shaken overnight. All the cells in suspension were then plated onto PDL coated coverslips. The percentage of each cell type was assessed before and after oligodendrocyte maturation. Live cells were stained with the specific microglial marker FITC-conjugated BSI-B<sub>4</sub> and the nuclear stains Hoechst and PI. The cells were viewed under the microscope and images were captured. The green fluorescent cells (FITC-BSI-B<sub>4</sub>) in each field were microglia. OPCs and oligodendrocytes did not stain with BSI-B<sub>4</sub> and were morphologically distinguished and counted. The experiments were repeated in quadruplicate and for each experiment at least three coverslips were viewed and at least four fields were counted per coverslip.

The co-cultures of microglia and OPCs after 1 DIV were found to contain 52.9 %  $\pm$  1.9 (SEM) microglia and 41 %  $\pm$  1.6 (SEM) OPCs. The ratio of microglia to OPCs was 1.41  $\pm$  0.16 (SEM). The co-cultures of microglia and 6 DIV mature oligodendrocytes (OLs) were found to contain 40.4 %  $\pm$  2 (SEM) microglia and 49.9 %  $\pm$  2.4 (SEM) oligodendrocytes. The ratio of microglia to mature oligodendrocytes was 0.9  $\pm$  0.1 (SEM). Representative images of co-cultures of microglia with OPCs or 6 DIV oligodendrocytes are shown in Figure 3.3.2.5.

The co-cultures were initially approximately 3:2 MG/OPCs and after oligodendrocyte maturation, the ratio decreased to approximately 2:3. This was most probably due to OPC proliferation. In the mouse brain cortex 5% of the cells were microglia (Lawson et al., 1990). OPCs comprise 5-8% of the CNS cell population (Levine et al., 2001). The ratio of microglia to mature oligodendrocytes was counted at approximately 2:3 in the rat corpus callosum (Miller et al., 2007). Thus, the composition of the co-cultures used was similar to the observed *in vivo* tissue composition. It should be noted that microglia and OPCs proliferate in response to brain injury, for example in acute lesion sites in MS (Solanky et al., 2001).





**Figure 3.4.1 Co-cultures of microglia and oligodendrocyte lineage cells**

Representative images of co-cultures plated on PDL coated glass coverslips, stained with FITC-conjugated BSI-B<sub>4</sub> (green) and the nuclear stains Hoechst (blue) and PI (red, dead cells). (A) are representative images of co-cultured OPCs and microglia without (i) or with (ii) phase contrast. (B) are representative images of co-cultured 6 DIV mature oligodendrocytes and microglia without (i) or with (ii) phase contrast. It was noticed that near mature oligodendrocytes the microglia do not retain the ramified morphology they typically present in culture and acquire a more activated morphology. Scale bar = 20  $\mu$ m.



### 3.5 Discussion

Collectively, for the study of the interactions between microglia and oligodendrocytes, a method for the isolation of pure microglia, OPCs and mature oligodendrocytes from 5 day old Sprague Dawley rats was developed. This method allowed for the best use of most brain tissue from each animal sacrificed (the cerebellum was also utilised for cerebellar granule cell culture). The main advantage of this method is that it is an easy and efficient method that produces sufficient numbers of all three glial cell populations. When required, pure cultures or co-cultures of the different brain glial cells were used to study different hypotheses. The purity of the cell cultures used and the composition of the co-cultures is summarised in Table 3.5.1.

**A**

Cell culture type	Purity % +/- SEM
Microglia	96.5 +/- 0.5
OPCs	92.8 +/- 1.3
6 DIV OL	91.9 +/- 0.7

**B**

Cell culture type	Microglia % +/- SEM	OPCs or OLs % +/- SEM	MG / OPCs or OLs +/- SEM
Microglia - OPCs	52.9 +/- 1.9	41 +/- 1.6	1.4 +/- 0.2
Microglia - 6DIV OLs	40.5 +/- 2	49.9 +/- 2.4	0.9 +/- 0.1

**Table 3.5.1 Composition of *in vitro* cell cultures**

(A) Table of the purity of microglial, OPC and 6 DIV oligodendrocyte (6 DIV OL) cultures with standard error mean (SEM). (B) Table of the composition of the microglial - OPC and 6 DIV - OL co-cultures as well as the ratio of cells in co-culture with standard error mean (SEM).

#### **4. INVESTIGATION OF OLIGODENDROCYTE LINEAGE CELL VULNERABILITY TO LPS ACTIVATED MICROGLIA AND GLUTAMATE**

## 4.1 Introduction

In this chapter the effect of lipopolysaccharide (LPS) activated microglia on OPCs and mature oligodendrocytes *in vitro* was studied. The molecules secreted following microglial activation were investigated in an attempt to clarify the molecular pathways resulting in oligodendrocyte lineage cell demise after microglial activation with LPS. The effect of the excitotoxic neurotransmitter glutamate on cells of the oligodendrocyte lineage was also studied and compared with its effect on microglial and cerebellar granule cell (CGC) viability.

### 4.1.1 Microglial activation in multiple sclerosis

Microglia have been implicated in all stages of MS disease development (see 1.3.4), including serving as antigen-presenting cells during acute inflammation, clearing away damaged tissue and cells after relapses and contributing to the irreversible damage associated with the chronic phase of the disease (Dhib-Jalbut, 2007). It has even been proposed that microglial activation is the initial pathogenic event in MS, suggesting that the primary reaction in MS is initiated from within the nervous system rather than from an invasive immune attack across a compromised blood-brain barrier (reviewed by Gay, 2007a).

Whether microglia initiate or assist in the immune response of the nervous system against myelin, their implication as activated cells in MS is well documented. They persist in active MS lesions in an activated state (Lassmann, 2003) and produce cytokines such as IL-23 (Li et al., 2007). *In vitro*, following myelin phagocytosis microglia were found to secrete cytokines such as IL-6, IL-1 and TNF- $\alpha$  (Williams et al., 1994). After LPS activation *in vitro*, microglia were shown to secrete TNF- $\alpha$  and shed FasL (Taylor et al., 2005).

### 4.1.2 LPS induced iNOS expression in microglia

LPS is a major component of the outer membrane of Gram-negative bacteria. It is the prototypical pathogen-associated molecular pattern (PAMP) and it induces a strong immune response (Poltorak et al., 1998; Reimer et al., 2008). LPS binds the

CD14/TLR4/MD2 receptor complex, which promotes the secretion of pro-inflammatory cytokines in macrophages and microglia. LPS is widely used experimentally to induce microglial activation. Microglial activation *in vitro* results in changes in cell morphology (amoeboid rather than ramified), proliferation, migration and secretion of soluble factors such as cytokines (reviewed by Färber and Kettenmann, 2005). One common event of microglial activation is the upregulation of the inducible nitric oxide synthase (iNOS) gene and the expression of iNOS and subsequent release of nitric oxide (NO). NO reacts to form reactive nitrogen species such as peroxynitrite that can lead to oxidative stress and cytotoxicity. iNOS expression has been used as a marker of microglial activation both *in vivo* and *in vitro* (Colasanti et al., 1995), although it is not ubiquitous for microglial activation or toxicity (Taylor et al., 2002). Microglia have been shown to strongly express inducible nitric oxide synthase (iNOS) in chronic active MS lesions (Hill et al., 2004) as well as following activation *in vitro* (Hooper et al., 2005; Hooper and Pocock, 2007; Pinteaux-Jones et al., 2008).

#### **4.1.3 Effects of microglial activation on OPCs**

OPCs, the precursor cell 'pool' of the brain, give rise to new oligodendrocytes during remyelination in MS. OPCs migrate to sites of inflammation and remyelinate exposed axons during the remitting stage of MS. However, remyelination progressively fails in MS. In the progressive phase of the disease although OPCs migrate to the lesion sites, they are not able to differentiate into oligodendrocytes (reviewed by Franklin and Ffrench-Constant, 2008). OPCs have been reported to be sensitive to LPS activated MG and excitotoxicity (Zajicek et al., 1992; Lehnardt et al., 2002; Li et al., 2008). It was therefore investigated how activated microglia could lead to OPC toxicity. The signalling mechanisms by which activated MG were toxic to OPCs were also investigated. TNF- $\alpha$  and iNOS have both been suggested as potential factors released from microglia that can mediate oligodendrocyte death (Zajicek et al., 1992; Merrill et al., 1993; Li et al., 2005; Li et al., 2008). The implication of these substances in the toxic phenotype of microglia was investigated.

There is evidence suggesting that microglial mGluRs can be manipulated and have a modulating effect on microglial neurotoxicity (Taylor et al., 2002; 2003; 2005;

Pinteaux-Jones et al., 2008). mGluRs have been characterised in both microglia and OPCs (see 1.5.2). Therefore the regulation of LPS induced oligodendrocyte death by mGluR manipulation was studied. The ability of different agonists and antagonists for all three groups of mGluRs to modulate toxicity was studied. Further work on mGluR regulation of microglial activation is discussed in Chapter 6.

#### **4.1.4 Effects of microglial activation on mature oligodendrocytes**

Myelinating oligodendrocytes are a principal target of the immune attack in multiple sclerosis (reviewed by Compston and Coles, 2002). Infiltrating macrophages release cytotoxic cytokines, while autoreactive antibodies target proteins of the myelin sheath such as MOG, MBP, MAG and PLP. It is an interesting question whether the activated microglia present in MS lesions affect oligodendrocytes further and contribute to their death, especially in the chronic phase of MS. There is contrasting evidence as to whether activated microglia present in MS lesions affect mature oligodendrocytes and contribute to their death. LPS activated microglia in co-culture were found to induce oligodendrocyte death (Domercq et al. 2007), whilst in a different *in vitro* study LPS activated microglia were shown to be beneficial to mature oligodendrocyte survival (Miller et al., 2007). Thus, the effect of LPS activated microglia on mature oligodendrocytes cultured *in vitro* was investigated. TNF- $\alpha$  and NO released from microglia were also studied as potential factors contributing to microglial toxicity to mature oligodendrocytes.

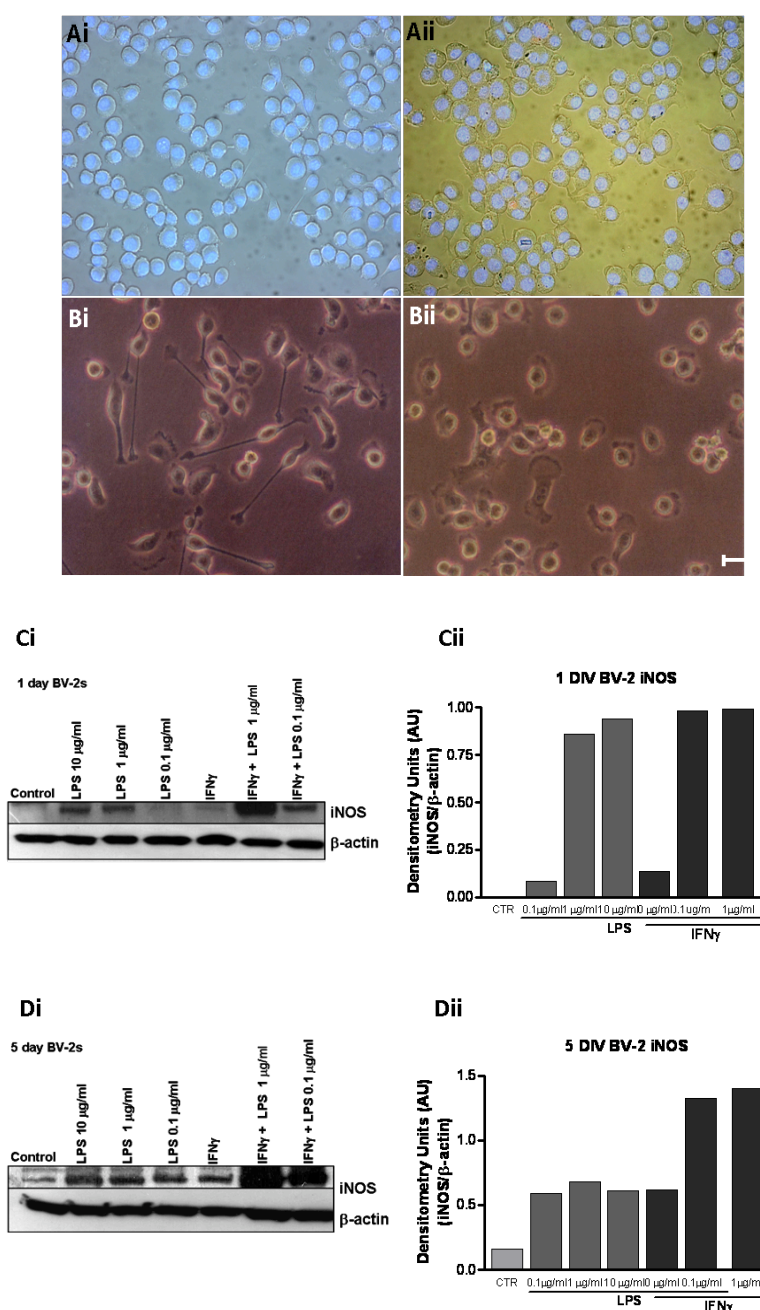
#### **4.1.5 Effects of glutamate on oligodendrocytes**

Glutamate is implicated in inducing excitotoxic death to all iGluR expressing neurones (reviewed by Lipton & Rosenberg, 1994). Based on reports that oligodendrocytes are also very vulnerable to increased concentrations of glutamate (Oka et al., 1993; Yoshioka et al., 1995; Matute et al., 1997; McDonald et al., 1998; Li & Stys, 2000) glutamate was used to induce oligodendrocyte death. The effect of glutamate on oligodendroglial cells of different developmental stages was studied and the results were compared with the effect of glutamate on microglia and cerebellar granule neurones (CGCs), an established model of excitotoxicity (Lowenstein et al., 1991).

## 4.2 LPS activation of microglia

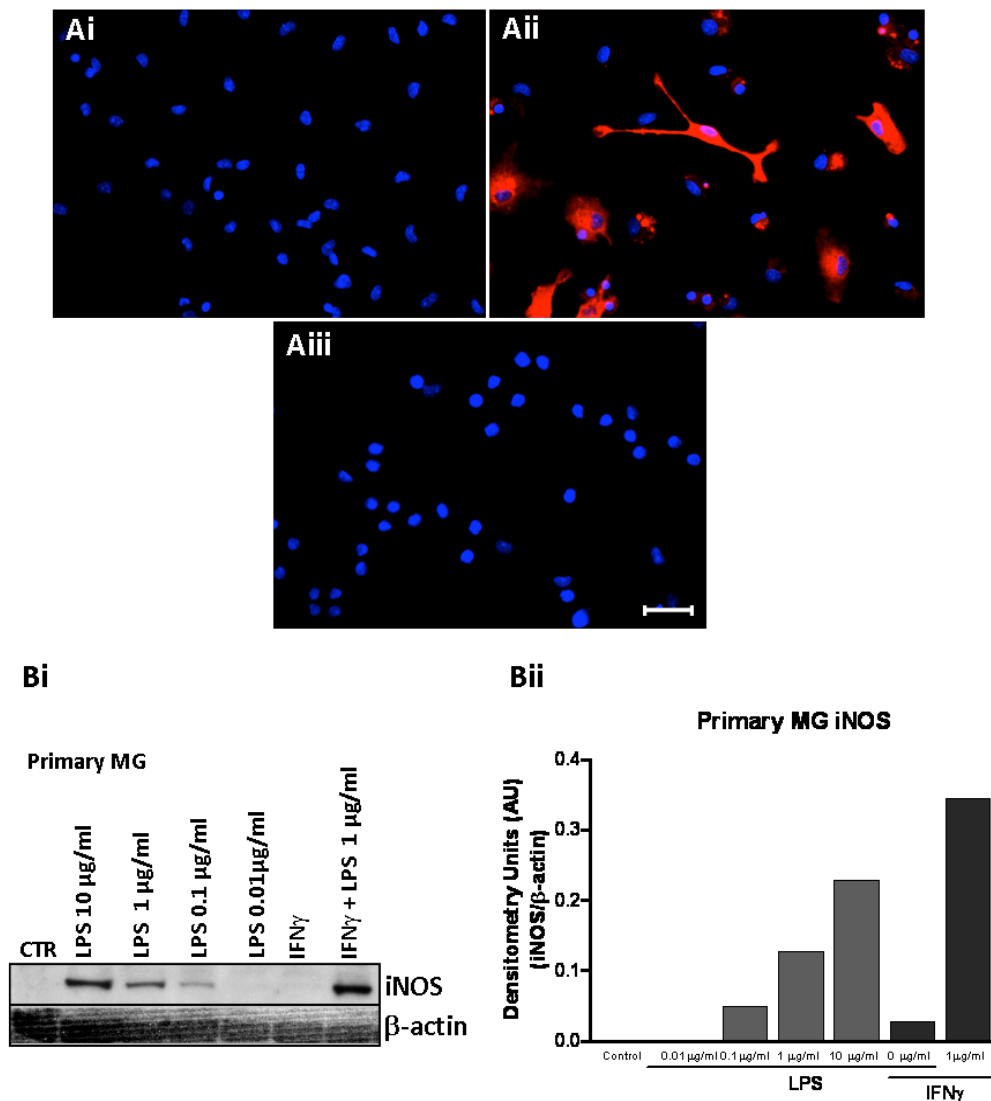
Here it was shown that LPS induced iNOS expression in the BV-2 cell line, as well as in primary microglia. Activated BV-2s were morphologically different from control cells (Figure 4.2.1 A,B). LPS induced iNOS expression in a dose-dependent way, and interferon- $\gamma$  (IFN $\gamma$ ), which is a cytokine physiologically secreted by T lymphocytes to activate microglial cells via their IFN- $\gamma$  receptors, enhanced LPS induced iNOS expression (Figure 4.2.1 C,D). BV-2s were cultured *in vitro* for 1 or 5 days and were then exposed to LPS for 24 hours. iNOS expression was observed in both cases. After 5 days *in vitro* untreated BV-2s exhibited basal iNOS expression, confirming that prolonged culture of the cell line, which was accompanied by proliferation and loss of ramified morphology due to space constriction, could potentially result in mechanisms that activated the cells in untreated cultures (Figure 4.2.1 D). Also, IFN $\gamma$  alone induced iNOS expression above basal levels in 5 day *in vitro* BV-2s. 1 day *in vitro* was chosen as the optimal time to use BV-2 cells for activation experiments.

LPS activation of primary microglia led to morphological changes and iNOS expression (Figure 4.2.2 A; Figure 4.2.2 B). The concentration of LPS required to induce iNOS expression in primary microglia was 0.1  $\mu\text{g/ml}$ , which was lower than the concentration required for BV-2 cell iNOS expression after 24 hour activation. IFN $\gamma$  upregulated iNOS expression following LPS activation of primary microglia. IFN $\gamma$  alone did not induce iNOS expression. 0.1 - 0.5  $\mu\text{g/ml}$  LPS were the concentrations selected to use in further experiments where microglial activation with LPS was required.



**Figure 4.2.1 iNOS expression in BV-2 cells activated with LPS**

A. Representative fluorescence microscopy images of control (i) and 1  $\mu\text{g/ml}$  LPS (ii) treated live BV-2 cells after 24 hours in culture. Nuclei were stained blue with Hoechst 33342 and a bright field phase contrast image was added. B. Representative optical microscopy images of control (i) and 1  $\mu\text{g/ml}$  LPS (ii) treated live BV-2 cells after 24 hours in culture. Scale bar = 20  $\mu\text{m}$ . C. Western blot (i) and densitometric analysis (ii) of iNOS expression of 1 day *in vitro* BV-2s after 24 hour activation. D. Western blot (i) and densitometric analysis (ii) of iNOS expression of 5 day *in vitro* BV-2s after 24 hour activation. LPS induced iNOS expression in a dose dependent way, whilst 100 U/ml interferon- $\gamma$  (IFN $\gamma$ ) enhanced this expression. IFN $\gamma$  alone induced iNOS expression above basal levels in 5 day *in vitro* BV-2s.  $\beta$ -actin was used as a loading control.



**Figure 4.2.2 iNOS expression in primary microglia activated with LPS**

A. Representative fluorescence microscopy images of fixed primary microglia in culture. (i) in control, (ii) in cells activated with 0.1  $\mu\text{g/ml}$  LPS for 24 hours, (iii) negative control (primary antibody omitted). Nuclei were stained blue with DAPI and the cytoplasm was stained red with anti-iNOS antibody. Scale bar = 20  $\mu\text{m}$ . B. Western blot (i) and densitometric analysis (ii) of iNOS expression in primary microglia (MG) after 24 hour activation. LPS induced iNOS expression in a dose dependent way, whilst 100 U/ml interferon- $\gamma$  (IFN $\gamma$ ) enhanced this expression.  $\beta$ -actin was used as a loading control.



### 4.3 LPS activated microglia were toxic to OPCs in co-culture

0.1 µg/ml LPS was administered to microglia to study the effect of microglial activation on OPCs *in vitro*. Microglia were plated on 13 mm coverslips at 25,000 cells/coverslip and were treated for 24 hours *in vitro*. OPCs were then stained live with Hoechst and PI to assess cell viability. When microglia and OPCs were cultured on the same coverslips (referred to as MG-OPC co-cultures), the co-cultures were also stained with 2 µg/ml FITC conjugated isolectin BSI-B<sub>4</sub> for 2 hours to selectively stain microglia green (Streit and Kreutzberg, 1987) and allow them to be distinguished from OPCs when counting the cells. The experimental rationale is depicted in Figure 2.4.1.

OPCs were initially treated with direct administration of LPS to study whether LPS was directly toxic to OPCs. LPS was not toxic to OPCs *in vitro* (Figure 4.3.1 A). Subsequently untreated or LPS treated serum-free MGCM was added to OPCs at a 1:1 ratio to oligodendrocyte culture media. MGCM was not toxic to OPCs. LPS treated MGCM was protective to OPCs and enhanced their survival in *in vitro* conditions (Figure 4.3.1). This effect was attributed to soluble factors secreted by activated microglia, since serum-free medium alone or MGCM did not protect OPCs in culture. The same experiment was repeated using the BV-2 and CG-4 cell lines and it was shown that use of these two cell lines produced similar results to the primary cultures (Figure 4.3.2). Furthermore, not just LPS activated BV-2 medium but also untreated BV-2 conditioned medium was found to be protective to CG-4s, indicating that soluble factors responsible for OPC survival could also be secreted from non-activated cells.

The next step was to culture microglia in inserts above cultured OPCs. The inserts had 0.4 µm pores that only allowed soluble molecules to pass through them. Thus the cells were compartmentalised without being in contact, whilst sharing the same medium. Microglia on inserts (20,000 cells/insert) were treated with 0.1 mg/ml LPS. Untreated microglia did promote OPC survival, although not significantly (Figure 4.3.3). LPS activated microglia did induce a small but statistically significant elevation of OPC cell death when compared with non-treated microglia (Figure 4.3.3).

Since MGCM did not induce OPC death and compartmentalisation of the cells resulted in only a small percentage of OPC death, the next step was to allow physical contact of the two cell populations. Thus, microglia and OPCs were co-cultured at a 3:2 ratio of MG to OPCs (see 3.4 for details). The co-cultures were treated with LPS. LPS activated microglia induced significant (\*\*\*)  $p < 0.001$  OPC death (Figure 4.3.4). This microglial-induced OPC death was attenuated by the selective inhibitor of tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ) synthesis, thalidomide (Thal) (Peterson et al., 1995). Thal suppresses TNF- $\alpha$  production by accelerating the degradation of TNF- $\alpha$  mRNA transcripts (Moreira et al., 1993). Thal inhibits TNF- $\alpha$  and IL-8 expression but does not affect the expression of other microglial cytokines released following microglial activation (Lokensgard et al., 2000). Microglia in the presence of Thal acquired a rounded activated morphology (Figure 4.3.4 B) but TNF- $\alpha$  was not secreted (Figure 4.3.7). Thus, OPC death was shown to be dependent on TNF- $\alpha$  production by microglia and required contact between the two cell populations.

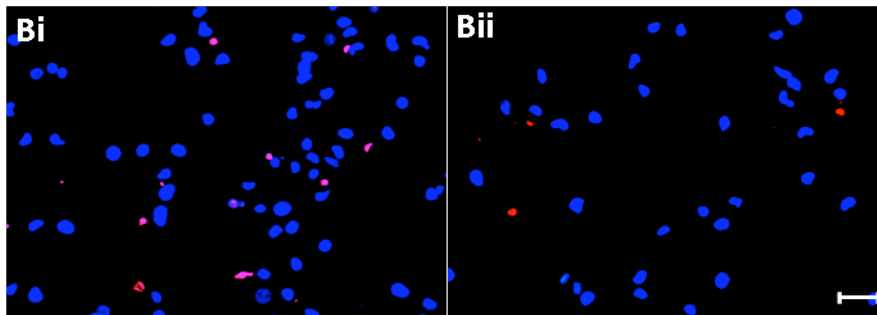
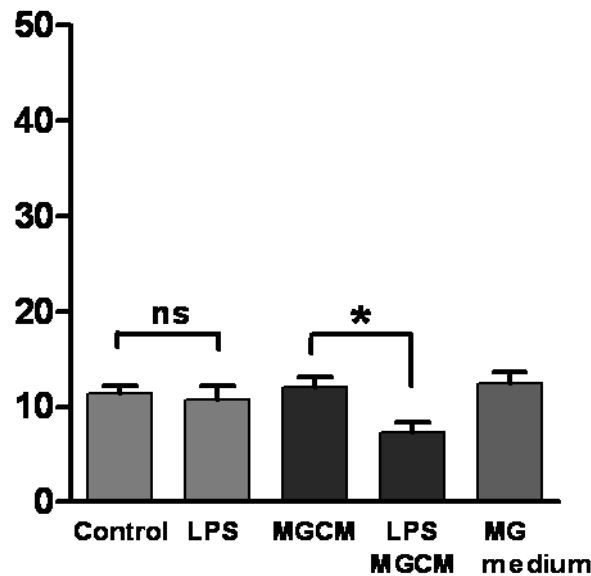
After 6 DIV, OPCs normally differentiated into myelin-expressing oligodendrocytes (see 3.3.3). It was tested whether TNF- $\alpha$  inhibition in the MG-OPC co-cultures would be able to abrogate the deleterious effect of LPS activated microglia to OPCs when the cells were cultured in contact for longer than 24 hours and thus would allow the cells to properly differentiate. Indeed, as opposed to LPS activation of microglia, which resulted in complete elimination of the OPC population within 6 days, TNF- $\alpha$  inhibition allowed OPCs to develop into mature oligodendrocytes in the same way as control cells when OPCs were cultured in contact with microglia (Figure 4.3.5).

TNF- $\alpha$  release by microglia in all the above experimental settings was measured using an ELISA kit (see 2.8). LPS activated microglia alone, in inserts above OPCs, or in co-culture produced similar amounts of TNF- $\alpha$ . The presence of 6 DIV oligodendrocytes under inserts with microglia or in co-culture with microglia also did not affect TNF- $\alpha$  release from LPS activated microglia (Figure 4.3.6). In all cases control microglia did not release TNF- $\alpha$ . The ability of thalidomide to block TNF- $\alpha$  release by LPS activated microglia was also measured. When thalidomide was added to LPS treated co-cultures, TNF- $\alpha$  release was attenuated, in agreement with the cell viability experiments (Figure 4.3.7).

In an attempt to block microglial-induced OPC death, the selective iNOS inhibitor AMT-HCl was added to the MG-OPC co-cultures 1 hour prior to LPS administration. AMT-HCl is a competitive inhibitor of L-arginine binding to iNOS. L-arginine is the NO substrate, thus no NO can be enzymatically produced (Nakane et al., 1995). iNOS was expressed by primary microglia *in vitro* in response to LPS activation (Figure 4.2.2) and since it has been implicated in neurotoxic cascades (Pinteaux-Jones et al., 2008) following microglial activation it was detected whether it had an active role in microglial-induced oligodendrocyte death. AMT-HCl was not directly toxic to the co-cultured OPCs and microglia (Figure 4.3.8 A). Incubation with increasing concentrations of AMT-HCl did not block LPS induced OPC death and thus cells did not differentiate into myelinating oligodendrocytes *in vitro* (figure 4.3.8 B). 3-morpholino-sydnonimine hydrochloride (SIN-1), that is an NO and superoxide releasing compound (de Groot et al., 1993) was used as an NO donor at 10 or 20  $\mu$ M (Fay et al., 2006) to further investigate whether OPCs were directly killed by NO, superoxide and peroxynitrite release. SIN-1 was added to OPCs for 24 hours and cell death was assessed by live staining. SIN-1 did not alter basal OPC death levels (Figure 4.3.9).

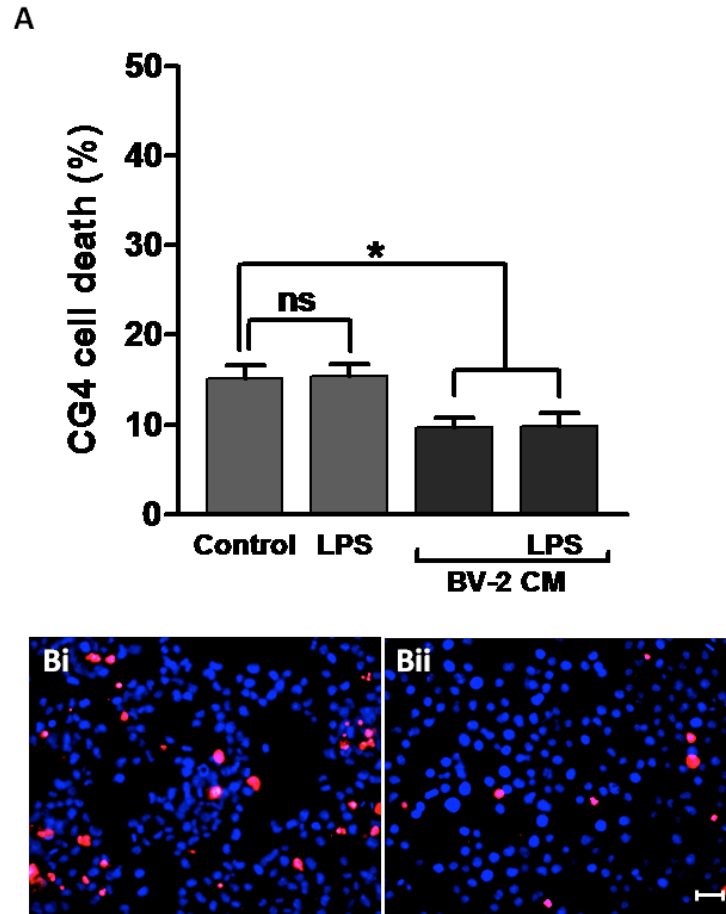
Modulation of the microglial induced OPC death was attempted by incubating the co-cultures with specific agonists and antagonists of mGlu receptors 30 minutes prior to LPS administration. The cells were then incubated for up to 6 DIV. The mGluR agonists and antagonists used were the group I agonist DHPG (100  $\mu$ M), the group I antagonists AIDA (250  $\mu$ M) and SIB-1757 (50  $\mu$ M) (mGlu5 specific), the group II agonists DCGIV (500 nM) and NAAG (50  $\mu$ M) (mGlu3 specific), the group II antagonist APICA (200  $\mu$ M), the group III agonists L-AP4 (100  $\mu$ M) and RSPPG (100  $\mu$ M), or the group III antagonist MAP4 (500  $\mu$ M). None of these agonists or antagonists were shown to be able to modulate microglial induced OPC death (Figure 4.3.10).

A



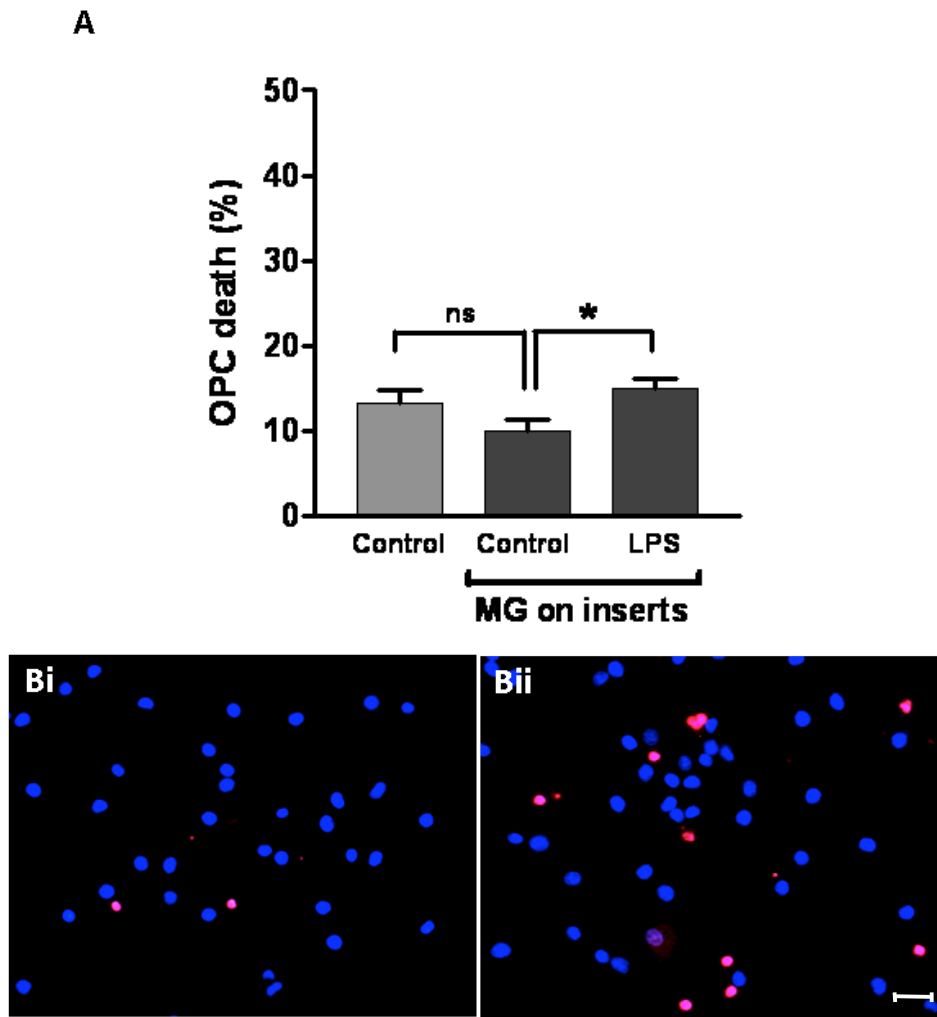
**Figure 4.3.1 LPS MGCM was protective to OPCs *in vitro***

A. OPCs were incubated with 50% MGCM for 24 hours and cell viability was assessed by live-dead staining. Cells were treated directly with 0.1  $\mu\text{g/ml}$  LPS, serum free unconditioned microglial media, conditioned MGCM, or MGCM treated with 0.1  $\mu\text{g/ml}$  LPS. Direct addition of LPS to OPCs was not cytotoxic. MGCM did not induce a cytotoxic phenotype either; whilst LPS treated MGCM was protective to OPCs. This effect was attributed to soluble factors secreted by activated microglia, since unconditioned media alone or MGCM did not protect OPCs. B. Representative fluorescence microscopy images of cell viability with MGCM (i) and LPS treated MGCM (ii). All nuclei were stained with Hoechst (blue), whilst late apoptotic and necrotic nuclei were stained with PI (red). Scale bar = 20  $\mu\text{m}$ . The values shown represent the mean  $\pm$  SEM of three experiments where three fields of each coverslip were counted. One way ANOVA with Tukey's post test was performed. Levels of significance were ns  $p>0.05$ , \*  $p<0.05$ .



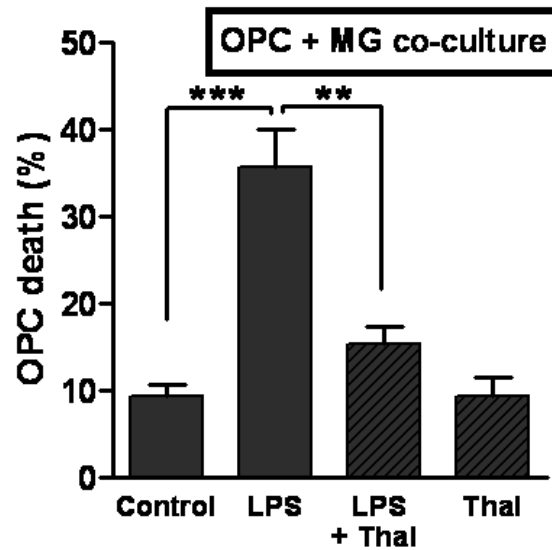
**Figure 4.3.2 BV-2 cell conditioned medium enhanced CG-4 cell survival**

A. CG4 cells were incubated with 50% control BV-2 CM, 50% BV-2 CM from 1  $\mu\text{g}/\text{ml}$  LPS treated BV-2 cells or directly with LPS for 24 hours and cell viability was assessed by live-dead staining. Control BV-2 CM or BV-2 CM activated with LPS was protective to CG-4 cells. This effect was attributed to soluble factors secreted by BV-2 cells in the conditioned medium. B. Representative fluorescence microscopy images of CG-4 cell viability of control cells (i) and cells incubated with BV-2 CM (ii). All nuclei were stained with Hoechst (blue), whilst late apoptotic and necrotic nuclei were stained with PI (red). Scale bar = 20  $\mu\text{m}$ . The values shown represent the mean  $\pm$  SEM of three experiments where three fields of each coverslip were counted. One way ANOVA with Tukey's post test was performed. Levels of significance were ns  $p > 0.05$ , \*  $p < 0.05$ .



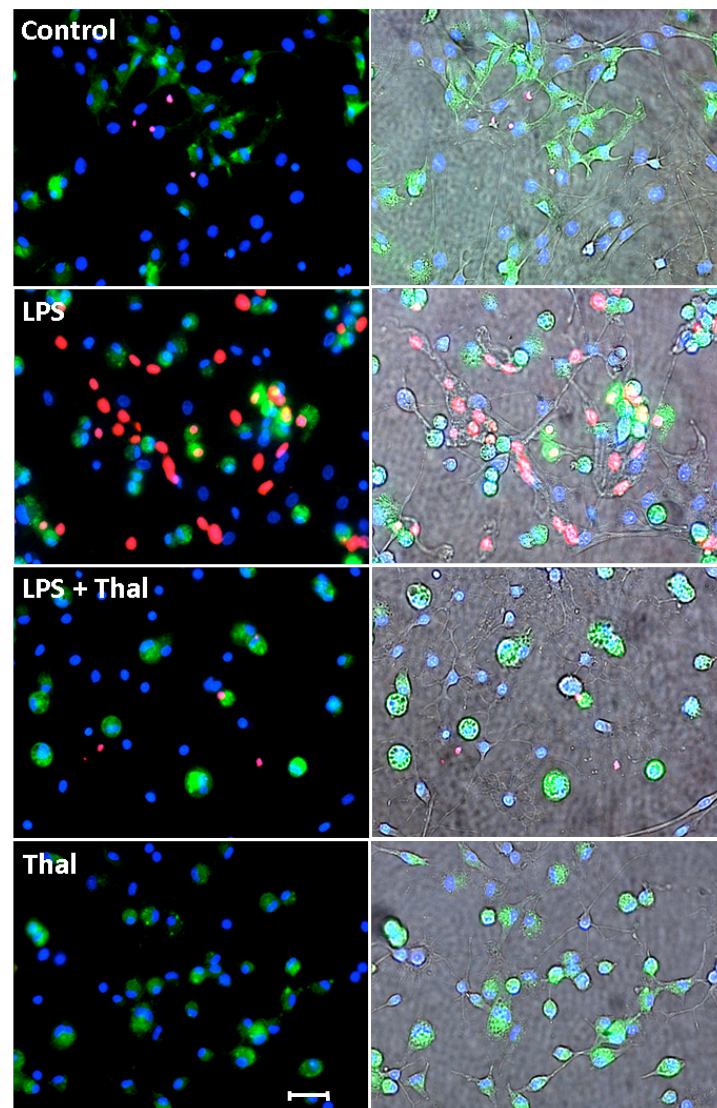
**Figure 4.3.3 LPS activated microglia on inserts were toxic to OPCs *in vitro***

A. OPCs were cultured alone (25,000 cells/well) or with addition of microglia (25,000 cells/insert) in 0.4  $\mu\text{m}$  pore cell inserts above the OPCs for 24 hours and cell viability was assessed by live-dead staining. Microglia on inserts were left untreated or treated with 0.1  $\mu\text{g/ml}$  LPS. The LPS activated microglia did induce a small but statistically significant elevation of OPC cell death when compared to non-treated microglia. B. Representative fluorescence microscopy images of cell viability of OPCs in the presence of untreated microglia on inserts (i) or LPS treated microglia on inserts (ii). All nuclei were stained with Hoechst (blue), whilst late apoptotic and necrotic nuclei were stained with PI (red). Scale bar = 20  $\mu\text{m}$ . The values shown represent the mean  $\pm$  SEM of three experiments where three fields of each coverslip were counted. One way ANOVA with Tukey's post test was performed. Levels of significance were ns  $p>0.05$ , \*  $p<0.05$ .



**Figure 4.3.4 A LPS activated microglia were toxic to OPCs when cultured in contact**

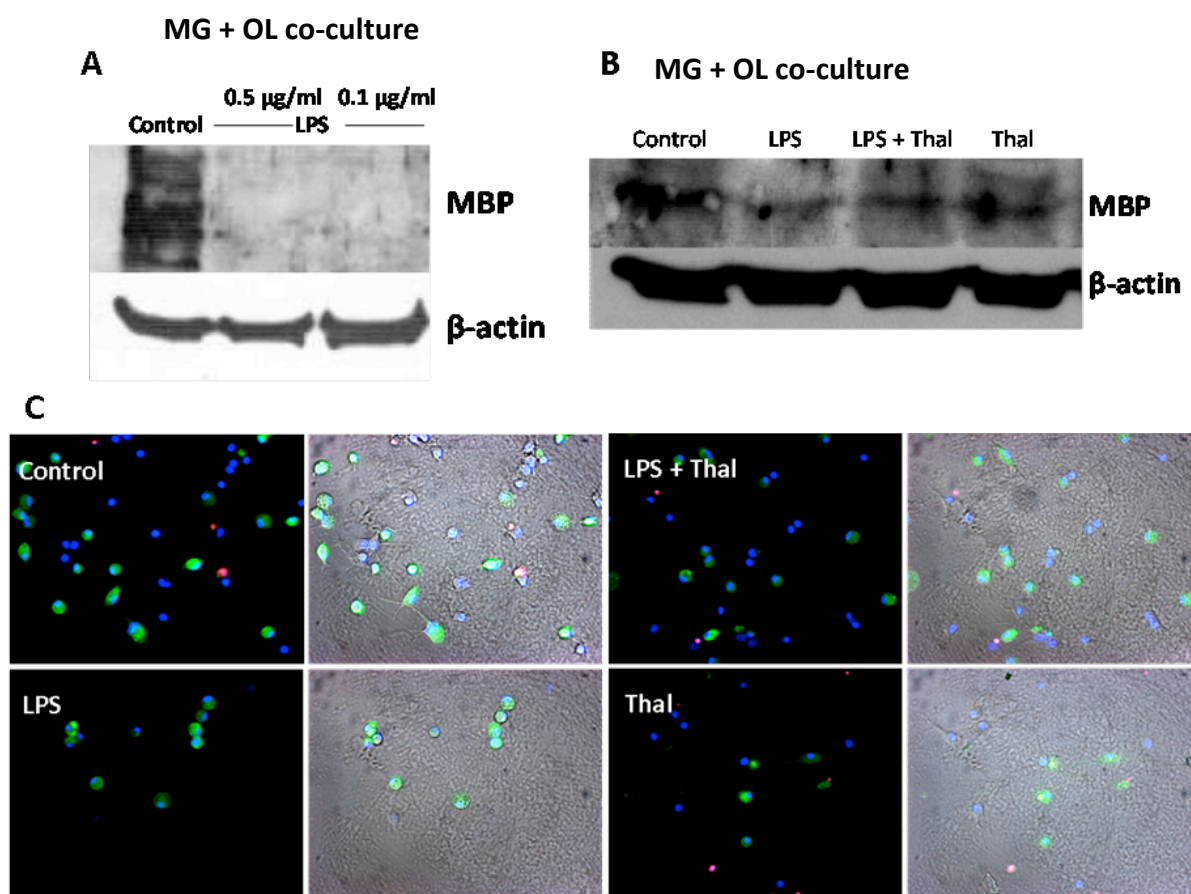
Microglia and OPCs were co-cultured in contact at a ratio of approximately 3:2 for 24 hours and cell viability was assessed by live-dead staining. The MG-OPC co-cultures were treated with 0.1 mg/ml LPS, 10  $\mu$ g/ml of the TNF- $\alpha$  synthesis inhibitor thalidomide (Thal) or both. LPS activated microglia induced significant OPC cell death when compared with non-treated OPC death levels in contact with microglia. Thalidomide was able to block microglial induced OPC death when the two cell populations were cultured together. The values shown represent the mean  $\pm$  SEM of three experiments where three fields of each coverslip were counted. One way ANOVA with Tukey's post test was performed. Levels of significance were \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .



**Figure 4.3.4 B LPS activated microglia were toxic to OPCs when cultured in contact**

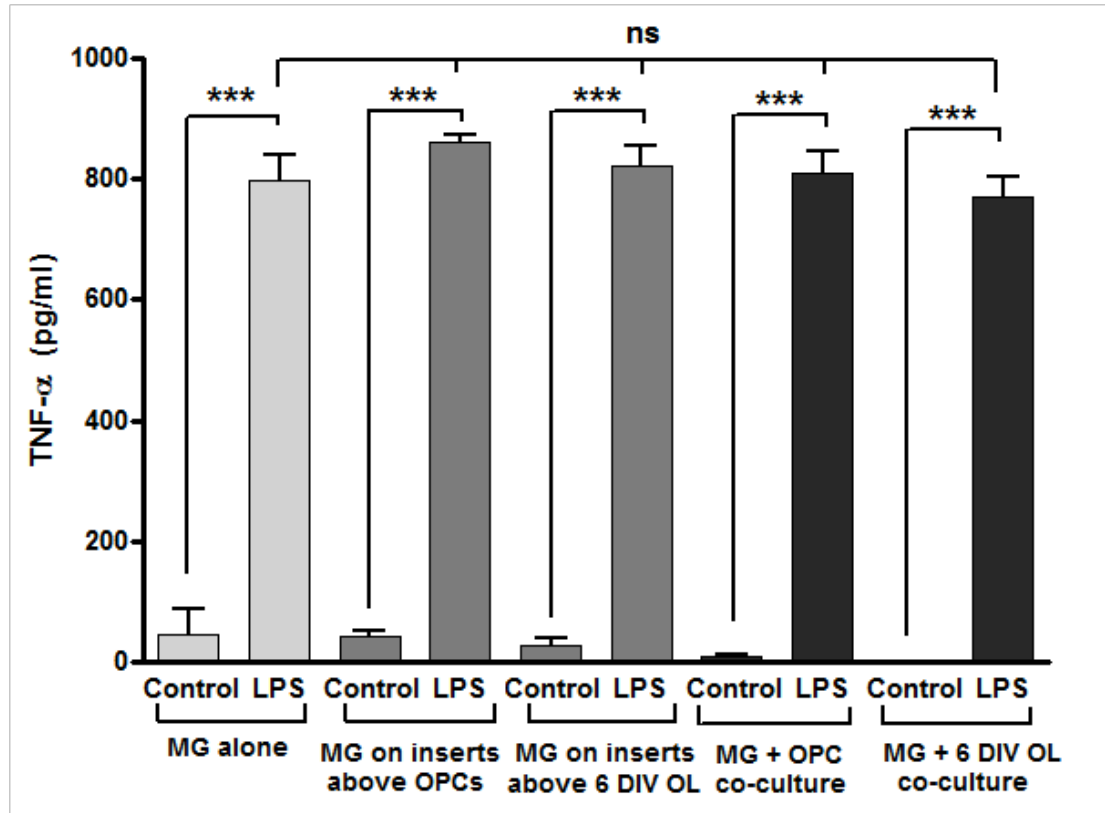
Microglia and OPCs were co-cultured in contact at a ratio of approximately 3:2. The MG-OPC co-cultures were pre-treated with 10  $\mu\text{g/ml}$  of the TNF- $\alpha$  synthesis inhibitor thalidomide (Thal), followed by 0.1 mg/ml LPS for 24 hours. LPS activated microglia induced significant OPC cell death when compared with non-treated co-culture OPC death levels. Thalidomide was able to block microglial induced OPC death when the two cell populations were cultured in contact. Representative fluorescence microscopy images of cell viability of OPCs in co-culture with microglia without or with phase contrast. All nuclei were stained with Hoechst (blue), whilst late apoptotic and necrotic nuclei were stained with PI (red). Isolectin BSI-B<sub>4</sub> was used to selectively stain microglia green and distinguish them from OPCs. The images shown are representative of three experiments. Scale bar = 20  $\mu\text{m}$ .





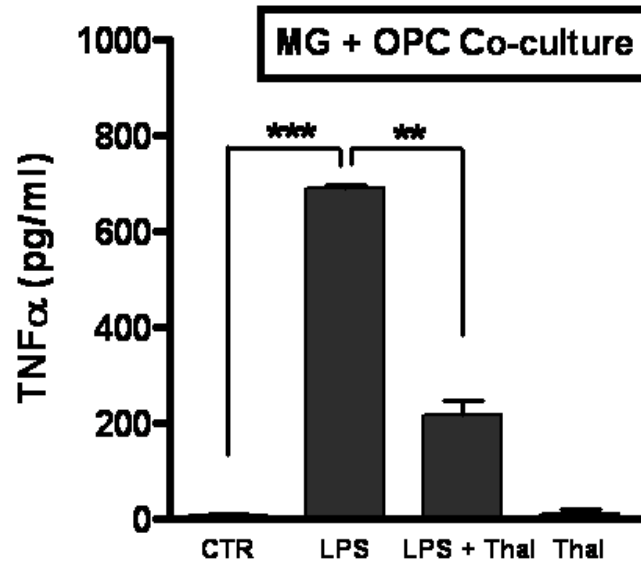
**Figure 4.3.5 Thalidomide facilitated OPC differentiation in the presence of LPS activated microglia**

A. Microglia and OPCs were co-cultured in contact at a ratio of approximately 3:2 for 6 days and were treated pre-treated with 10  $\mu\text{g/ml}$  of the TNF- $\alpha$  synthesis inhibitor thalidomide (Thal) followed by 0.1 or 0.5  $\mu\text{g/ml}$  LPS for the whole period of the 6 days. LPS activated microglia were toxic to OPCs and killed them before they were able to differentiate. Thalidomide blocked the microglial induced OPC death. A. Western blot of MBP expression in the co-cultures after 6 DIV. LPS activated microglia killed the OPCs before they could differentiate into oligodendrocytes. B. Western blot of MBP expression in the co-cultures after 6 DIV. Thalidomide blocked microglial induced OPC toxicity and subsequently the OPCs differentiated into oligodendrocytes. C. Representative fluorescence microscopy images of co-cultures without or with phase contrast. All nuclei were stained with Hoechst (blue), whilst late apoptotic and necrotic nuclei were stained with PI (red). Microglia were stained with their selective marker isolectin BSI-B<sub>4</sub> (green). Images represent experiments repeated in triplicate. Scale bar = 20  $\mu\text{m}$ .



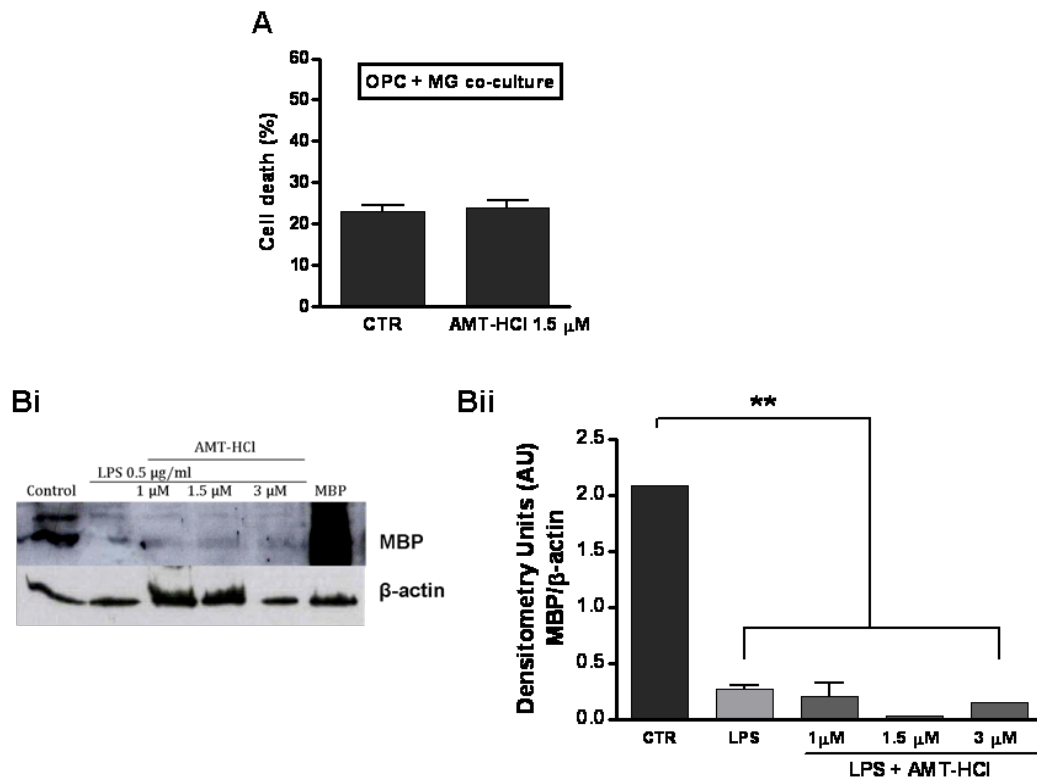
**Figure 4.3.6 The presence of OPCs or 6 DIV oligodendrocytes did not affect TNF- $\alpha$  release from LPS activated microglia**

Microglia were cultured alone, in inserts above OPCs or 6 DIV oligodendrocytes, or in co-culture in contact with OPCs or 6 DIV oligodendrocytes at a ratio of approximately 3:2 for 24 hours. Microglia were activated with 0.1  $\mu\text{g/ml}$  LPS and TNF- $\alpha$  release was measured with an ELISA kit. LPS activated microglia alone, in inserts, or in co-culture produced TNF- $\alpha$ . TNF- $\alpha$  production was statistically significant in all cases, while there were no statistical significances between the different control and LPS treated samples. The values shown represent the mean  $\pm$  SEM of three experiments where each sample was assessed in duplicate. One way ANOVA with Tukey's post test was performed. Levels of significance were ns  $p > 0.05$ , \*\*\*  $p < 0.001$ .



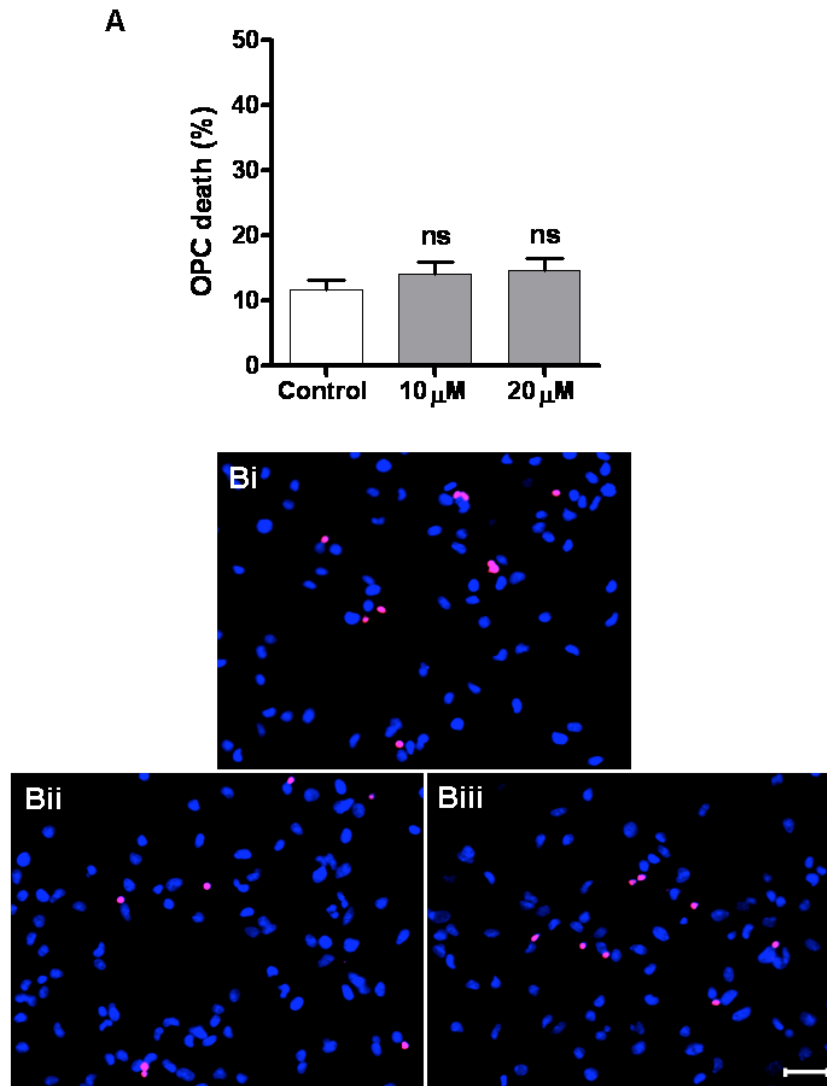
**Figure 4.3.7 Thalidomide attenuated TNF- $\alpha$  release from LPS activated microglia in co-culture with OPCs**

Microglia and OPCs were co-cultured at a ratio of approximately 3:2 for 24 hours. Microglia were activated with 0.1 mg/ml LPS. The TNF- $\alpha$  synthesis inhibitor thalidomide (Thal, 10  $\mu$ g/ml) was used to block TNF- $\alpha$  production. An ELISA kit was used to measure TNF- $\alpha$  release. Thalidomide attenuated TNF- $\alpha$  release when added to LPS treated microglia in contact with OPCs. Thalidomide alone did not induce any TNF- $\alpha$  release. The values shown represent the mean  $\pm$  SEM of three experiments where each sample was assessed in duplicate. One way ANOVA with Tukey's post test was performed. Levels of significance were ns  $p > 0.05$ , \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .



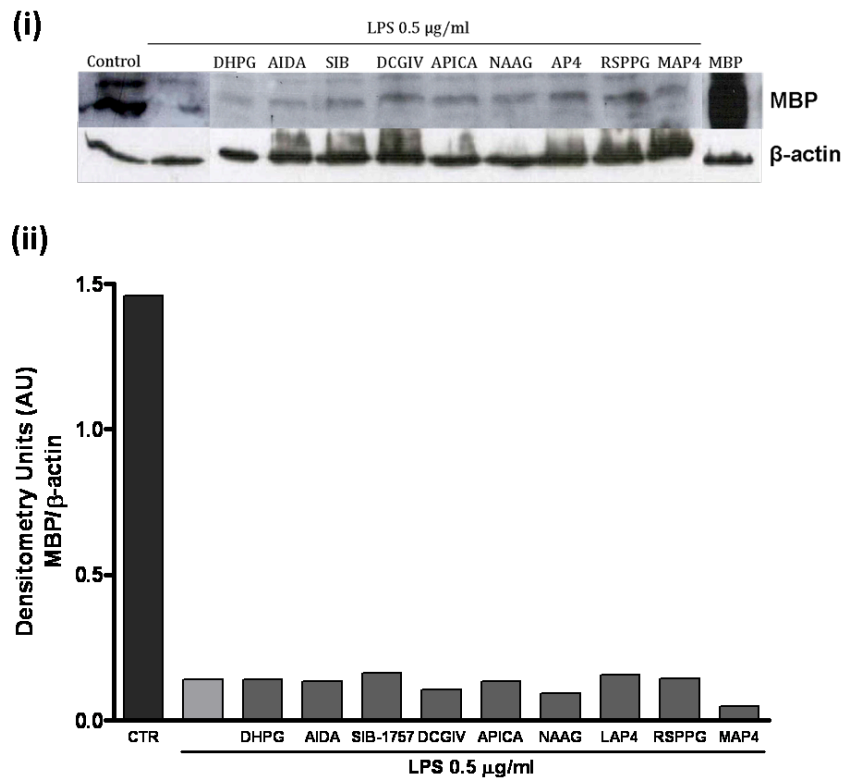
**Figure 4.3.8 iNOS inhibition could not modulate microglial induced OPC death**

A. Microglia and OPCs were co-cultured in contact at a ratio of approximately 3:2 for 24 hours and cell viability was assessed by live-dead staining. The co-cultures were treated with 1.5 mM of the specific iNOS inhibitor AMT-HCl. AMT-HCl was not toxic to the cells. The values shown represent the mean  $\pm$  SEM of three experiments where three fields of each coverslip were counted. B. Representative western blot (i) and densitometric analysis (ii) of MBP expression in co-cultures of primary OPCs and microglia after 6 DIV. LPS activated microglia induced OPC death, thus MBP was only expressed in control cells after 6 DIV. Increasing concentrations of AMT-HCl were unable to modulate microglial induced OPC death. The blots shown are representative of three experiments. For the densitometric semi-quantifying analysis one way ANOVA with Tukey's post test was performed. Levels of significance were ns  $p > 0.05$ , \*\*  $p < 0.01$ .



**Figure 4.3.9 NO was not directly toxic to OPCs**

A. OPCs were cultured alone (25,000 cells/well) for 24 hours and cell viability was assessed by live-dead staining. OPCs were treated with 10 or 20  $\mu$ M of the NO and superoxide donor SIN-1. SIN-1 was not toxic to the cells. B. Representative fluorescence microscopy images of cell viability of control (i), 10  $\mu$ M SIN-1 (ii) and 20  $\mu$ M SIN-1 (iii) treated OPCs. I nuclei were stained with Hoechst (blue), whilst late apoptotic and necrotic nuclei were stained with PI (red). Scale bar = 20  $\mu$ m. The values shown represent the mean  $\pm$  SEM of three experiments where three fields of each coverslip were counted. One way ANOVA with Tukey's post test was performed. Level of significance was ns  $p>0.05$ .



**Figure 4.3.10 Metabotropic glutamate receptors could not modulate microglial induced OPC death**

Representative western blot (i) and densitometric analysis (ii) of MBP expression in co-cultures of primary OPCs and microglia after 6 DIV. 0.5  $\mu\text{g/ml}$  LPS was used to activate the microglia and induce OPC death. LPS was also used in conjunction with the mGluR agonists and antagonists 100  $\mu\text{M}$  DHPG, 250  $\mu\text{M}$  AIDA, 50  $\mu\text{M}$  SIB-1757, 500 nM DCGIV, 200  $\mu\text{M}$  APICA, 50  $\mu\text{M}$  NAAG, 100  $\mu\text{M}$   $\text{lAP4}$ , 100  $\mu\text{M}$  RSPPG or 500  $\mu\text{M}$  MAP4. The mGluR agonists and antagonists were not able to modulate the toxic effect of LPS on OPCs, thus MBP was only expressed in control cells after 6 DIV. Purified MBP was used as a positive control. The experiment shown is representative of four experiments performed where oligodendrocyte cell death was visually confirmed but western blotting for MBP did not always result in visible bands, thus a representative blot was analysed and presented.

#### 4.4 LPS activated microglia were toxic to 6 DIV oligodendrocytes in co-culture

To study the effect of microglial activation on mature oligodendrocytes, 6 DIV oligodendrocytes were chosen as a model of mature oligodendrocytes *in vitro*. At that stage myelin proteins such as MBP and MOG were expressed (Figure 3.3.2.3). Later than 6 DIV, oligodendrocytes progressively started dying so the basal levels of death were high (>30%) and experimental results were not reliable. LPS at (0.1 – 0.5) µg/ml was administered to microglia to induce their activation and study the effect of their activation to 6 DIV mature oligodendrocytes *in vitro*. Cells were treated with LPS for 24 hours and then stained live with Hoechst and PI to assess cell viability. When co-cultured, the cells were stained with isolectin BSI-B<sub>4</sub> to selectively stain microglia green and to distinguish them from oligodendrocytes when counting the cells. The experimental rationale is depicted in Figure 2.4.1.

6 DIV oligodendrocytes were initially treated by direct administration of 0.5 µg/ml LPS. LPS was not toxic to 6 DIV oligodendrocytes *in vitro* (Figure 4.4.1 A). Subsequently, untreated or 0.5 µg/ml LPS treated serum free MGCM was added to 6 DIV oligodendrocytes at a 1:1 ratio to oligodendrocyte culturing media. MGCM was not toxic or protective to 6 DIV oligodendrocytes (Figure 4.4.1).

The next step was to culture microglia on 0.4 µm pore inserts above cultured 6 DIV oligodendrocytes. The inserts allowed soluble molecules to pass through them. Microglia on inserts were treated with 0.1 mg/ml LPS. Untreated or LPS treated microglia were not protective nor toxic to 6 DIV oligodendrocytes (Figure 4.4.2).

To study the effect of microglia on 6 DIV oligodendrocytes when the cells were in contact, 5,000 microglia or 20K microglia were added for 24 hours on coverslips where 25,000 6 DIV oligodendrocyte cultures had already matured. 0.5 µg/ml LPS or 1 mM glutamate or both were added directly to the 6 DIV oligodendrocytes or to the cultures where microglia were added (co-cultures). From this initial experiment it was observed that 20,000 microglia were able to promote 6 DIV oligodendrocyte survival *in vitro* (Figure 4.4.3). Addition of 20,000 microglia with LPS on 6 DIV oligodendrocytes resulted in oligodendrocyte death. 20,000 microglia rather than 5,000 microglia were thus selected as the optimal number of cells for the co-

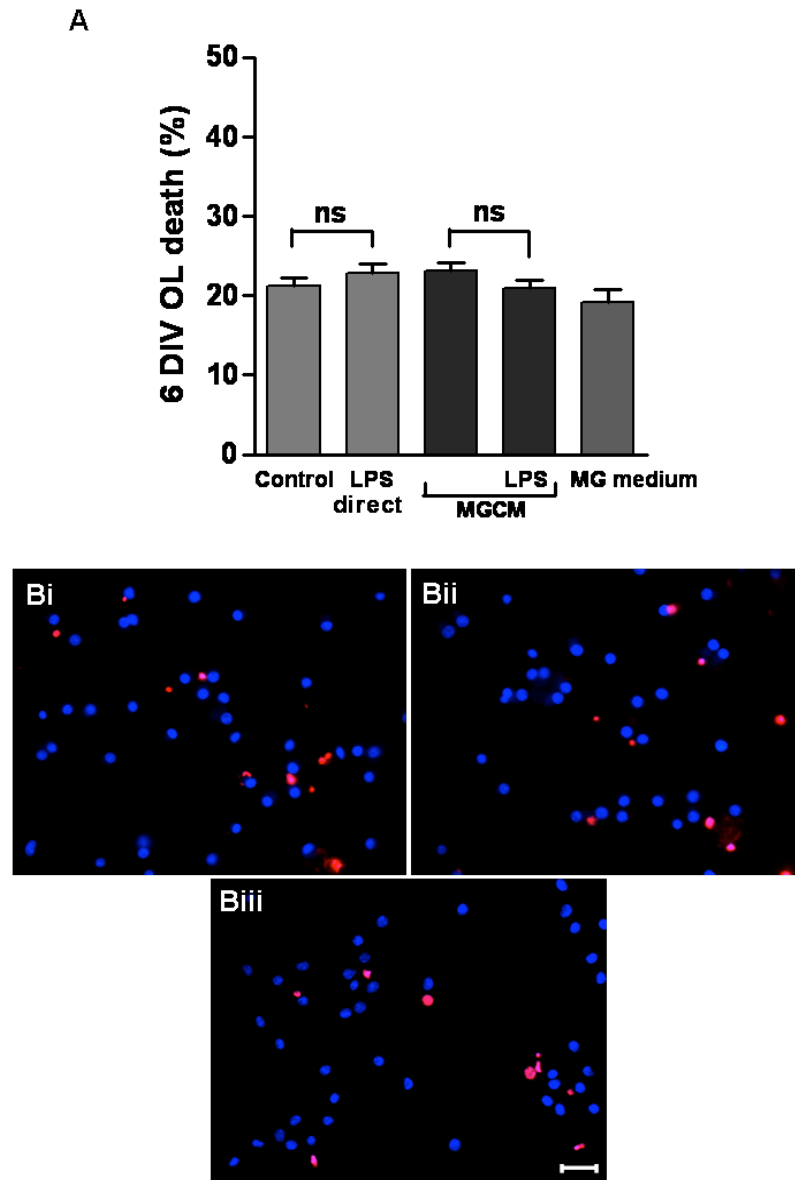
cultures. This ratio of cells (2:3 microglia : 6 DIV oligodendrocytes) could also be attained if the microglia and oligodendrocytes were plated and cultured together from 1 DIV (see 2.3.4). Thus co-culture of the cell populations directly after isolation of the cells was preferred, since this allowed less oligodendrocyte basal death (compare 4.4.3 to 4.4.4 A).

Conversely, the co-culture of 6 DIV oligodendrocytes and microglia in contact (hereafter referred to as MG-6 DIV OL co-culture) at a ratio of 3:2 (see 3.4 for details) resulted in oligodendrocyte toxicity when microglia were activated with LPS (Figure 4.4.4). Oligodendrocyte toxicity could be reversed by using 4  $\mu\text{g/ml}$  TNF- $\alpha$  synthesis inhibitor Thal (Figure 4.4.4). Thus, mature oligodendrocyte death, like OPC death, was attenuated when the cells were incubated with Thal and occurred only when the two cell populations were co-cultured. As shown earlier TNF- $\alpha$  release from microglia was measured with an ELISA kit in all the experimental settings used (Figure 4.3.6). As with the microglial and OPC co-cultures, in all experimental settings control microglia did not release TNF- $\alpha$ , while LPS activated microglia released equal amounts of TNF- $\alpha$ . The ability of Thal to attenuate TNF- $\alpha$  release by LPS activated microglia was also measured. When Thal was added to LPS treated MG - 6 DIV OL co-cultures, TNF- $\alpha$  release was attenuated, in agreement with the cell viability experiments (Figure 4.4.5).

Oligodendrocyte death was also prevented if the co-cultures were treated with 1 mM glutamate (Figure 4.4.6). 1 mM glutamate is a physiologically non-relevant concentration since even in excitotoxic conditions extracellular glutamate concentration does not exceed a concentration of 100  $\mu\text{M}$ . This result was attributed to the fact that glutamate overload impaired microglial function and thus TNF- $\alpha$  was not produced. Glutamate did not induce mature oligodendrocyte toxicity, as further analysed and experimentally evaluated in 4.5.

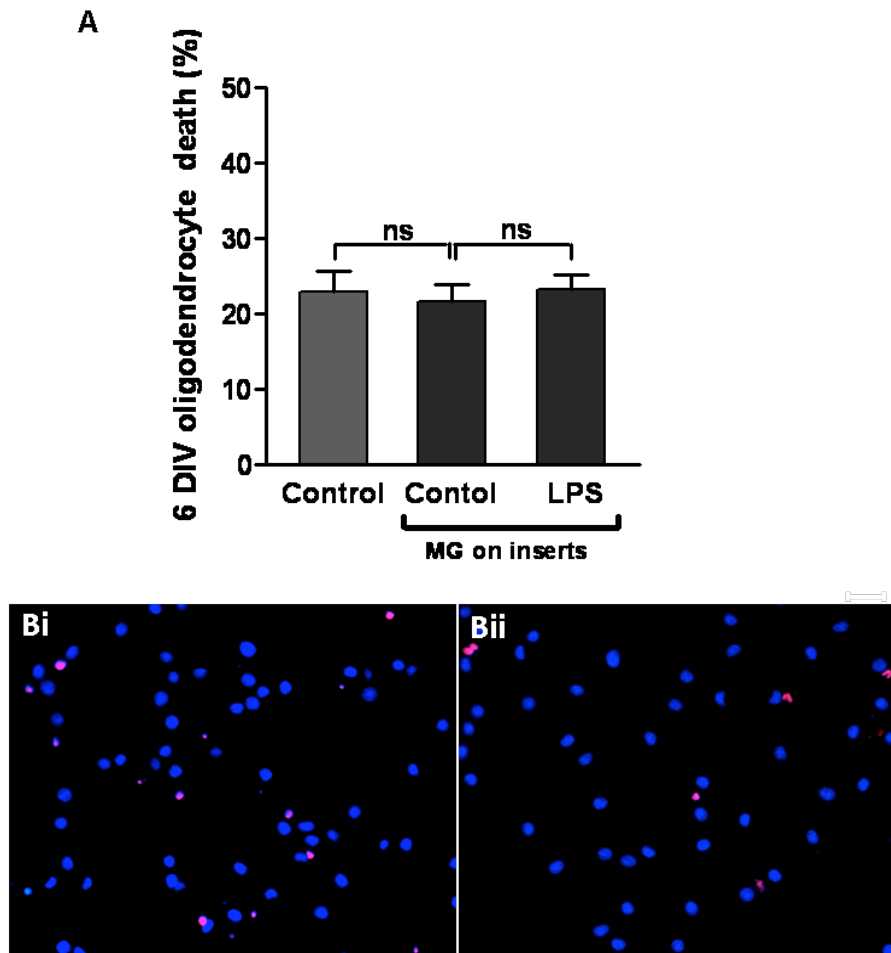
Finally, the NO and superoxide donor 3-morpholiniosydnonimine (SIN-1) was used at 10 or 20  $\mu\text{M}$  (Fay et al., 2006) to investigate whether 6 DIV oligodendrocytes were vulnerable to NO, superoxide or peroxynitrite release. SIN-1 was added to 6 DIV oligodendrocytes for 24 hours and cell death was assessed by live staining. SIN-1 did not alter basal 6 DIV oligodendrocyte death levels (Figure 4.4.7), suggesting that NO release by microglia or astrocytes is not implicated in oligodendrocyte death.





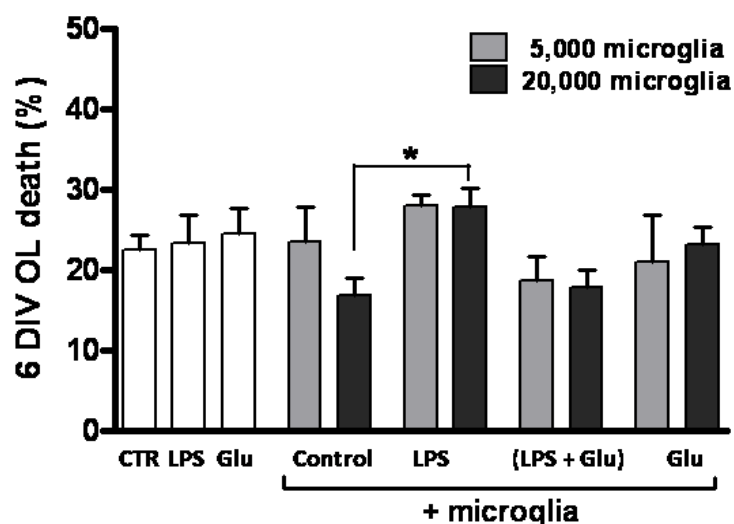
**Figure 4.4.1 MGCM was not toxic or protective to 6 DIV oligodendrocytes**

A. 6 DIV primary oligodendrocytes were incubated with 50% MGCM for 24 hours and cell viability was assessed by live-dead staining. Cells were treated with 0.5 mg/ml LPS, untreated MGCM or MGCM conditioned with LPS. Direct addition of LPS was not cytotoxic. MGCM with or without LPS activation did not affect cell viability. B. Representative fluorescence microscopy images of 6 DIV oligodendrocyte viability with MGCM (i) LPS conditioned MGCM (ii) and direct administration of LPS (iii). All nuclei were stained with Hoechst (blue), whilst late apoptotic and necrotic nuclei were stained with PI (red). Scale bar = 20  $\mu$ m. The values shown represent the mean  $\pm$  SEM of seven experiments where three fields of each coverslip were counted. One way ANOVA with Tukey's post test was performed. Level of significance was ns  $p>0.05$ .



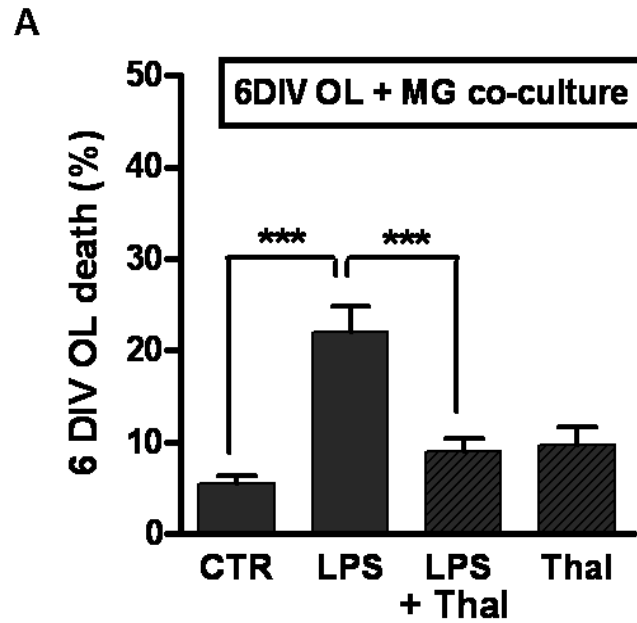
**Figure 4.4.2 LPS activated microglia on inserts were not toxic to 6 DIV oligodendrocytes**

A. 6 DIV oligodendrocytes were cultured alone (25,000 cells/well) or with addition of microglia (25,000 cells/insert) in 0.4  $\mu\text{m}$  pore cell inserts above the oligodendrocytes for 24 hours and cell viability was assessed by live-dead staining. Microglia on inserts were left untreated or treated with 0.1 mg/ml LPS. LPS treated microglia did not induce cytotoxicity. B. Representative fluorescent microscopy images of cell viability of 6 DIV oligodendrocytes in the presence of untreated microglia on inserts (i) or LPS treated microglia on inserts (ii). All nuclei were stained with Hoechst (blue), whilst late apoptotic and necrotic nuclei were stained with PI (red). Scale bar = 20  $\mu\text{m}$ . The values shown represent the mean  $\pm$  SEM of three experiments where three fields of each coverslip were counted. One way ANOVA with Tukey's post test was performed. Level of significance was ns  $p > 0.05$ .



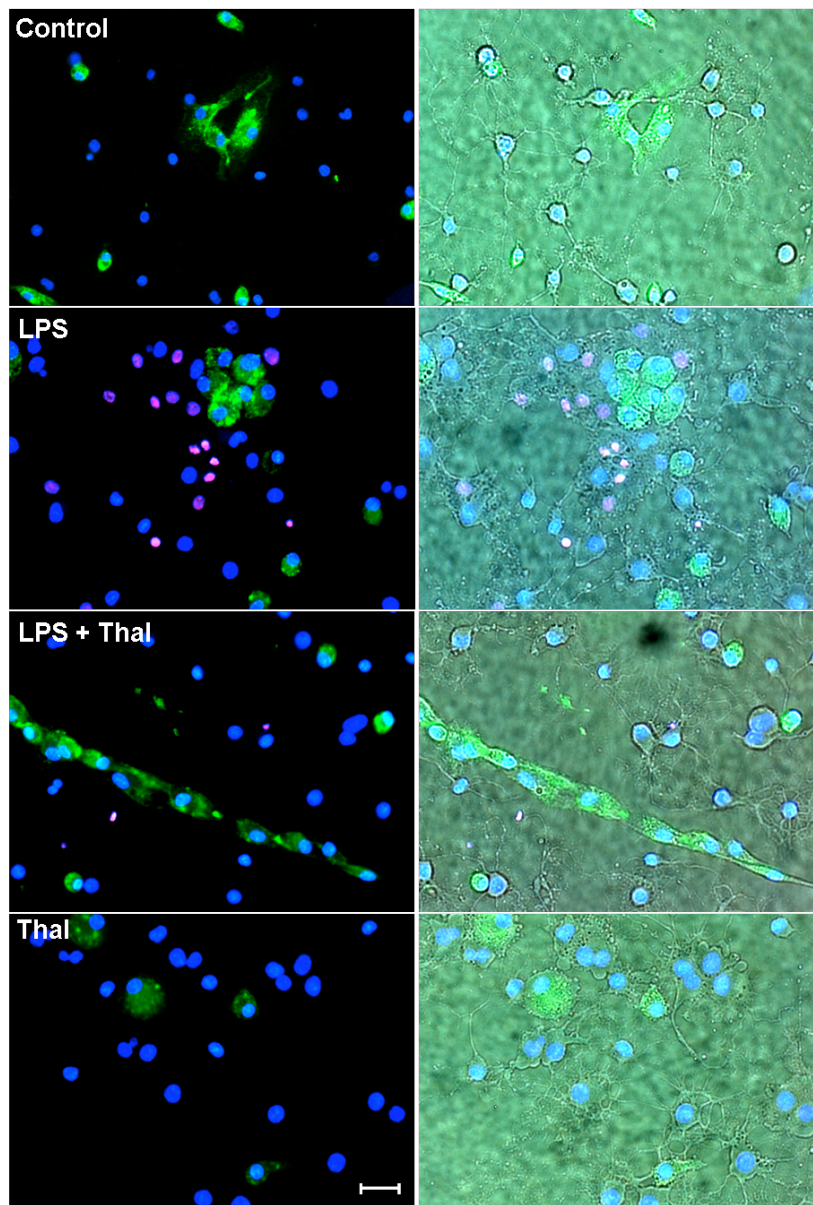
**Figure 4.4.3 LPS activated microglia were toxic to 6 DIV oligodendrocytes when co-cultured in contact**

5 microglia or 20K microglia were added for 24 hours on coverslips where 25K 6 DIV oligodendrocyte cultures had already matured and cell viability was assessed. 0.5  $\mu\text{g/ml}$  LPS or 1 mM glutamate or both were added directly to the 6 DIV oligodendrocytes or to the cultures where microglia were added (co-cultures). From this initial experiment it was observed that 20 K microglia were able to promote 6 DIV oligodendrocyte survival *in vitro*. Addition of 20K microglia with LPS on 6 DIV oligodendrocytes resulted in oligodendrocyte death. 20K microglia rather than 5K microglia were thus selected as the optimal number of cells for the co-cultures. This ratio of cells (2:3 microglia : 6 DIV oligodendrocytes) corresponded better to the ratio of the cell populations *in vivo*. Also, 20K microglia promoted 6 DIV oligodendrocyte viability and induced oligodendrocyte death more potently than 5K microglia when activated with LPS. A student's t test was performed to compare between two groups. Levels of significance were ns  $p>0.05$ , \*  $p<0.05$ .



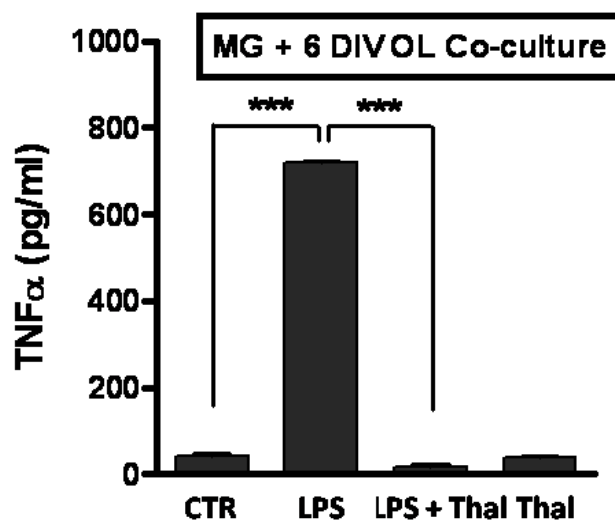
**Figure 4.4.4 A LPS activated microglial toxicity to 6 DIV oligodendrocytes was attenuated by TNF- $\alpha$  synthesis inhibition**

6 DIV oligodendrocytes and microglia were co-cultured at a ratio of 3:2 for 24 hours and cell viability was assessed by live-dead staining. The co-cultures were pre-treated with 4  $\mu\text{g/ml}$  TNF- $\alpha$  synthesis inhibitor thalidomide (Thal) for 30 minutes and then treated with 0.1  $\mu\text{g/ml}$  LPS. LPS activated microglia induced 6 DIV OL death. Thalidomide was able to block microglial induced 6 DIV oligodendrocyte death. The values shown represent the mean  $\pm$  SEM of three experiments where three fields of each coverslip were counted. One way ANOVA with Tukey's post test was performed. Level of significance was \*\*\*  $p < 0.001$ .



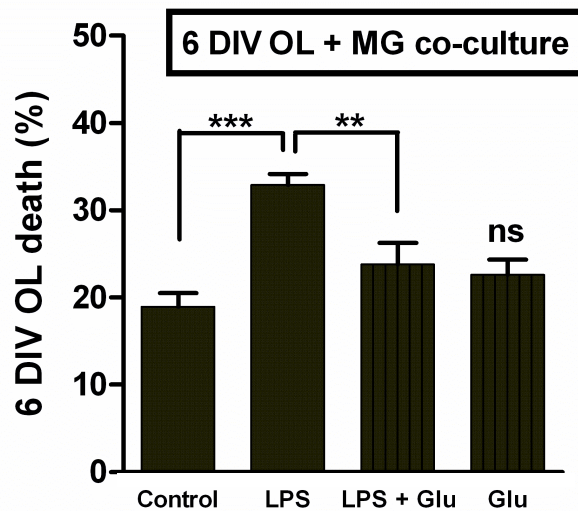
**Figure 4.4.4 B LPS activated microglial toxicity to 6 DIV oligodendrocytes was blocked by TNF- $\alpha$  synthesis inhibition**

6 DIV oligodendrocytes and microglia were co-cultured at a ratio of 3:2 for 24 hours. The co-cultures were pre-treated with 4  $\mu\text{g}/\text{ml}$  TNF- $\alpha$  synthesis inhibitor thalidomide (Thal) followed by 0.1  $\mu\text{g}/\text{ml}$  LPS. LPS activated microglia induced 6 DIV OL death. Thalidomide was able to block microglial induced 6 DIV oligodendrocyte death. Representative fluorescence microscopy images of cell viability of oligodendrocytes in co-culture without or with phase contrast are shown. All nuclei were stained with Hoechst (blue), whilst late apoptotic and necrotic nuclei were stained with PI (red). Isolectin BSI-B<sub>4</sub> was used to selectively stain microglia green and distinguish them from oligodendrocytes. Scale bar = 20  $\mu\text{m}$ . The values shown represent the mean  $\pm$  SEM of three experiments where three fields of each coverslip were counted. The images shown are representative of three experiments.



**Figure 4.4.5 Thalidomide attenuated TNF- $\alpha$  release from LPS activated microglia cultured in contact with 6 DIV oligodendrocytes**

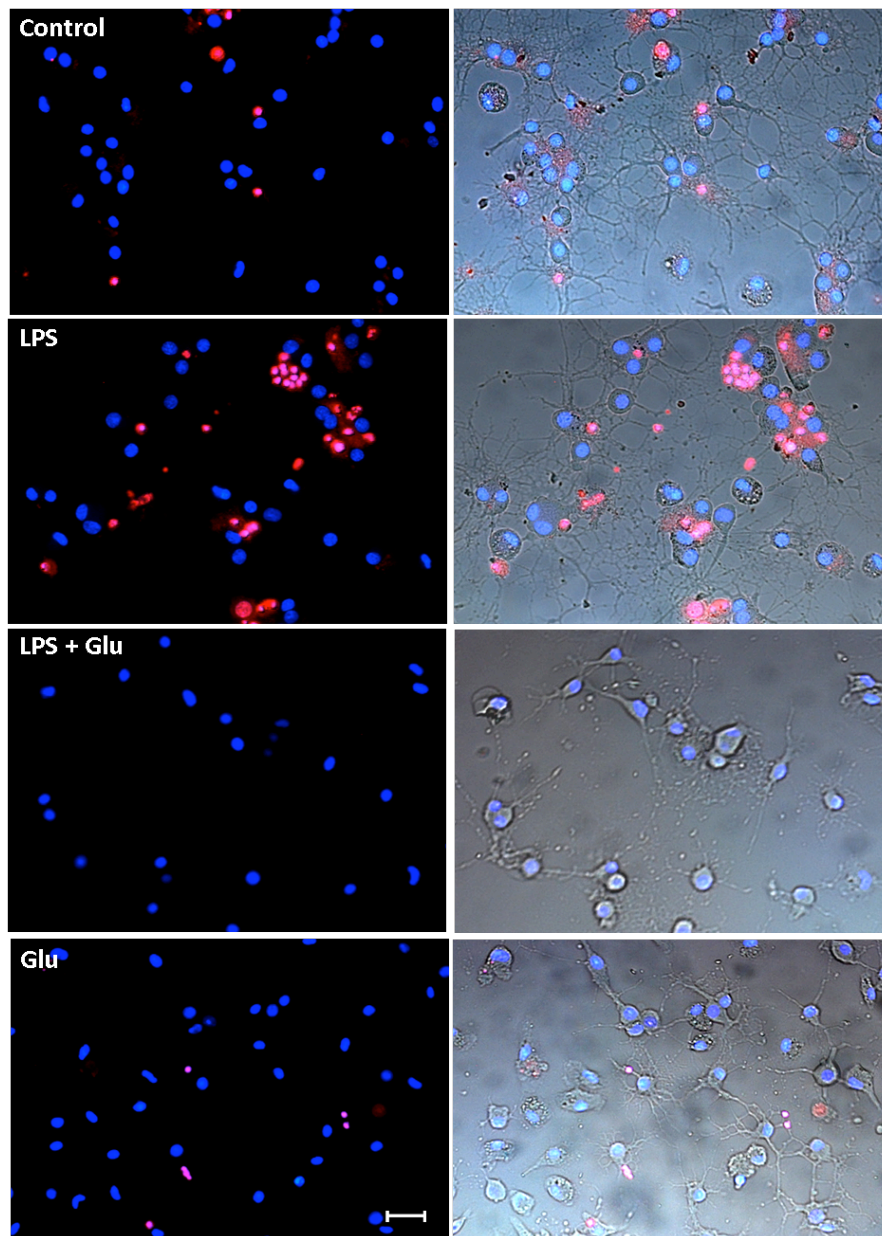
Microglia and 6 DIV oligodendrocytes were co-cultured at a ratio of approximately 2:3 for 24 hours. Microglia were activated with 0.1 mg/ml LPS. The TNF- $\alpha$  synthesis inhibitor thalidomide (Thal, 4  $\mu$ g/ml) was used to attenuate TNF- $\alpha$  production. An ELISA kit was used to measure TNF- $\alpha$  release. Thalidomide attenuated TNF- $\alpha$  release when added to LPS treated microglia in contact with 6 DIV oligodendrocytes. Thalidomide alone did not induce any TNF- $\alpha$  release. The values shown represent the mean  $\pm$  SEM of three experiments where each sample was assessed in duplicate. One way ANOVA with Tukey's post test was performed. Level of significance was \*\*\*  $p < 0.001$ .



**Figure 4.4.6 A Glutamate prevented LPS activated microglial-induced toxicity to 6 DIV oligodendrocytes**

Microglia were added on 6 DIV oligodendrocytes at a ratio of approximately 3:2 for 24 hours and cell viability was assessed by live-dead staining. The co-cultures were treated with 0.1 mg/ml LPS, 1 mM glutamate (Glu) or both. LPS activated microglia induced 6 DIV oligodendrocyte cell death. In the presence of glutamate LPS activated microglia did not induce 6 DIV oligodendrocyte death. Glutamate was not directly toxic to OL in co-culture. The values shown represent the mean  $\pm$  SEM of three experiments where three fields of each coverslip were counted. One way ANOVA with Tukey's post test was performed. Levels of significance were \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

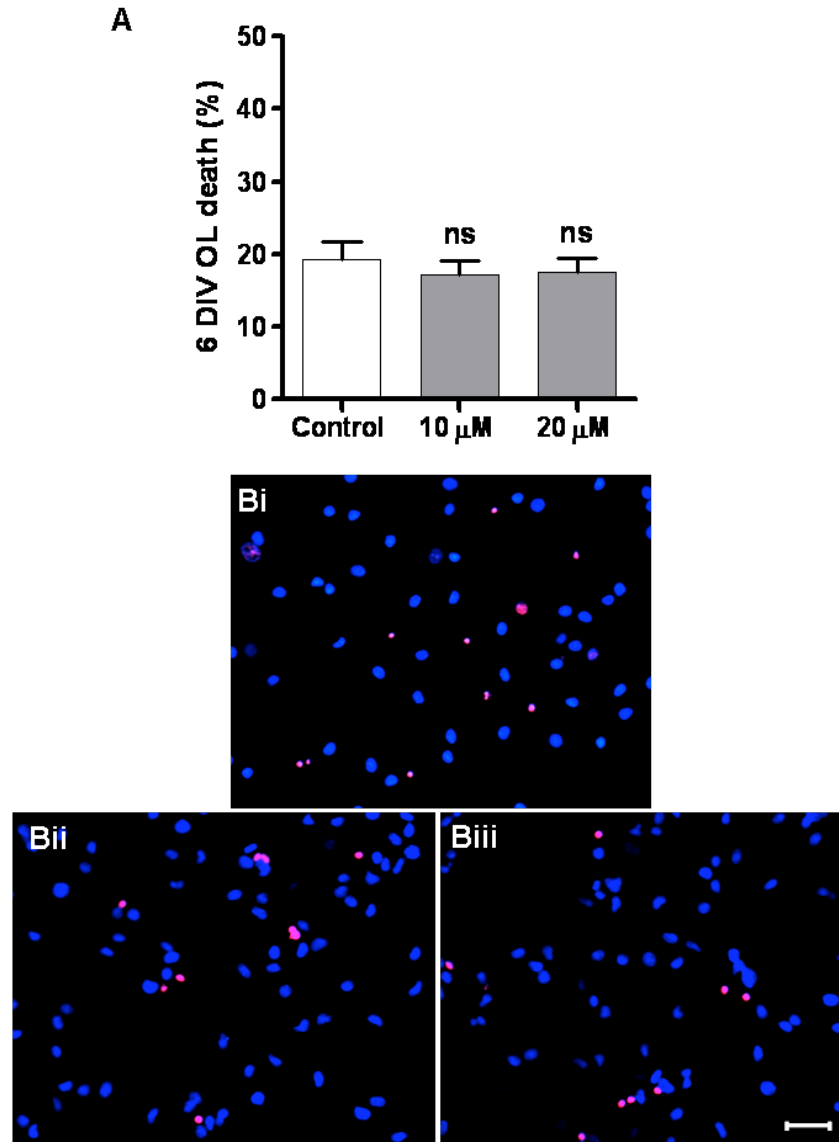




**Figure 4.4.6 B Glutamate prevented LPS activated microglial-induced toxicity to 6 DIV oligodendrocytes**

Microglia and 6 DIV oligodendrocytes were co-cultured at a ratio of approximately 2:3 for 24 hours. Representative fluorescent microscopy images of 6 DIV oligodendrocytes stained with Hoechst (blue) and PI (red) with or without phase contrast. Co-cultures were treated with 0.1 mg/ml LPS, 1 mM glutamate (Glu) or both. LPS activated microglia induced 6 DIV oligodendrocyte cell death. In the presence of glutamate LPS activated microglia were unable to induce 6 DIV oligodendrocyte death. Glutamate was not directly toxic to OL in co-culture. Scale bar = 20  $\mu$ m. The images shown are representative of three experiments.





**Figure 4.4.7 NO was not directly toxic to 6 DIV oligodendrocytes *in vitro***

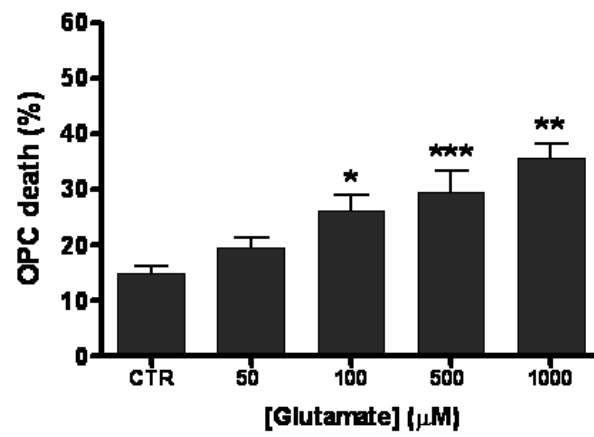
A. 6 DIV oligodendrocytes were cultured alone (25.000 cells/well) for 24 hours and cell viability was assessed by live-dead staining. 6 DIV oligodendrocytes were treated with 10 or 20  $\mu$ M of the NO and superoxide donor SIN-1. SIN-1 was not toxic to the cells. B. Representative fluorescence microscopy images of cell viability of control (i), 10  $\mu$ M SIN-1 (ii) and 20  $\mu$ M SIN-1 (iii) treated 6 DIV oligodendrocytes. Nuclei were stained with Hoechst (blue), whilst late apoptotic and necrotic nuclei were stained with PI (red). Scale bar = 20  $\mu$ m. The values shown represent the mean  $\pm$  SEM of three experiments where three fields of each coverslip were counted. One way ANOVA with Tukey's post test was performed. Level of significance was ns  $p > 0.05$ .

#### **4.5 Glutamate was toxic to OPCs and developing oligodendrocytes but not 6 DIV mature oligodendrocytes**

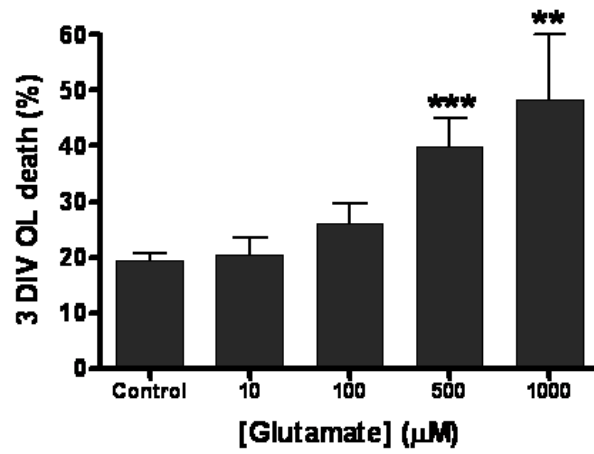
Glutamate was administered to oligodendroglial cells of different maturation stages in an attempt to clarify the effect of glutamate excitotoxicity on these cells. The effects of glutamate to oligodendroglial cells were compared with CGCs, an established model of excitotoxicity (Lowenstein et al., 1991), thus allowing the comparison of the results obtained for oligodendrocyte lineage cells with a 'positive' glutamate excitotoxicity model. The effects of glutamate to microglial cell toxicity were also studied. The glutamate solution was made fresh in distilled, sterilised water on the day of the experiment and cells were incubated with increasing concentrations for 24 hours, after which they were stained live with Hoechst and PI to assess cell viability.

Glutamate was significantly toxic to OPCs above 100  $\mu$ M in a concentration dependent manner (Figure 4.5.1 A,B). 3 DIV developing oligodendrocytes were vulnerable to glutamate above 500  $\mu$ M in a concentration dependent manner. (Figure 4.5.1 A,B). 6 DIV oligodendrocytes were not vulnerable to glutamate at any concentration used (Figure 4.5.1. A,B). CGCs were vulnerable to glutamate in a concentration dependent manner and the plotted graph was used to compare the effects of glutamate on CGCs with glutamate toxicity to oligodendrocytes (Figure 4.5.2). Microglia were activated following incubation with glutamate as assessed by upregulated ED1 staining and morphological changes but no toxicity to the cells was observed at 24 hours *in vitro* (Figure 4.5.3).

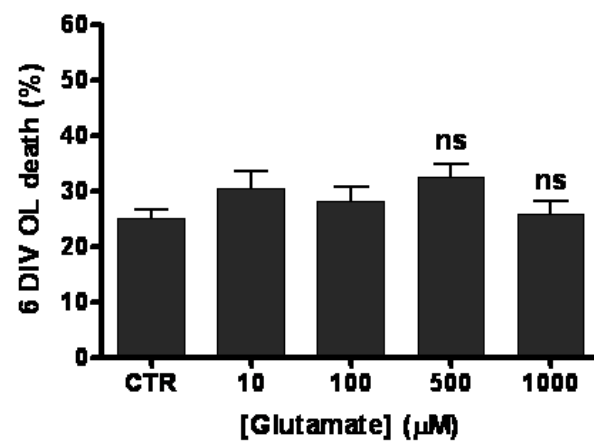
**Ai**

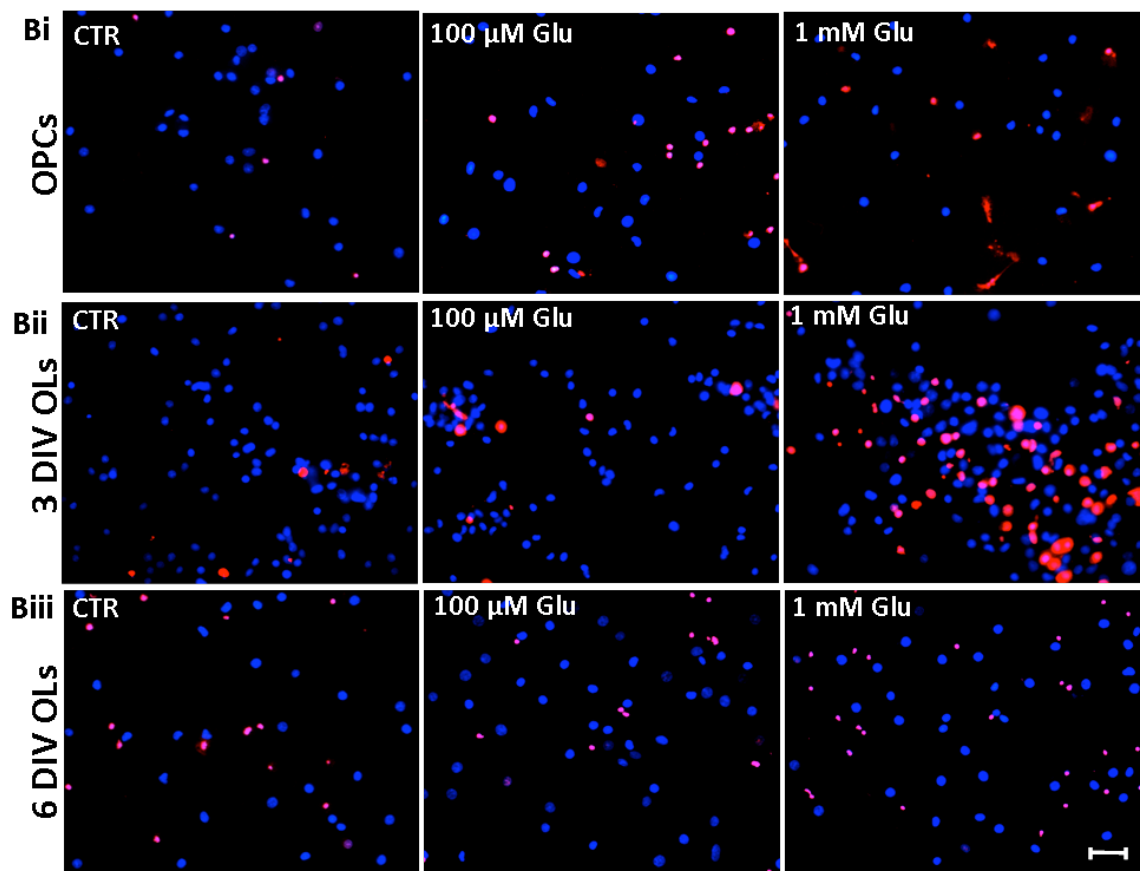


**Aii**



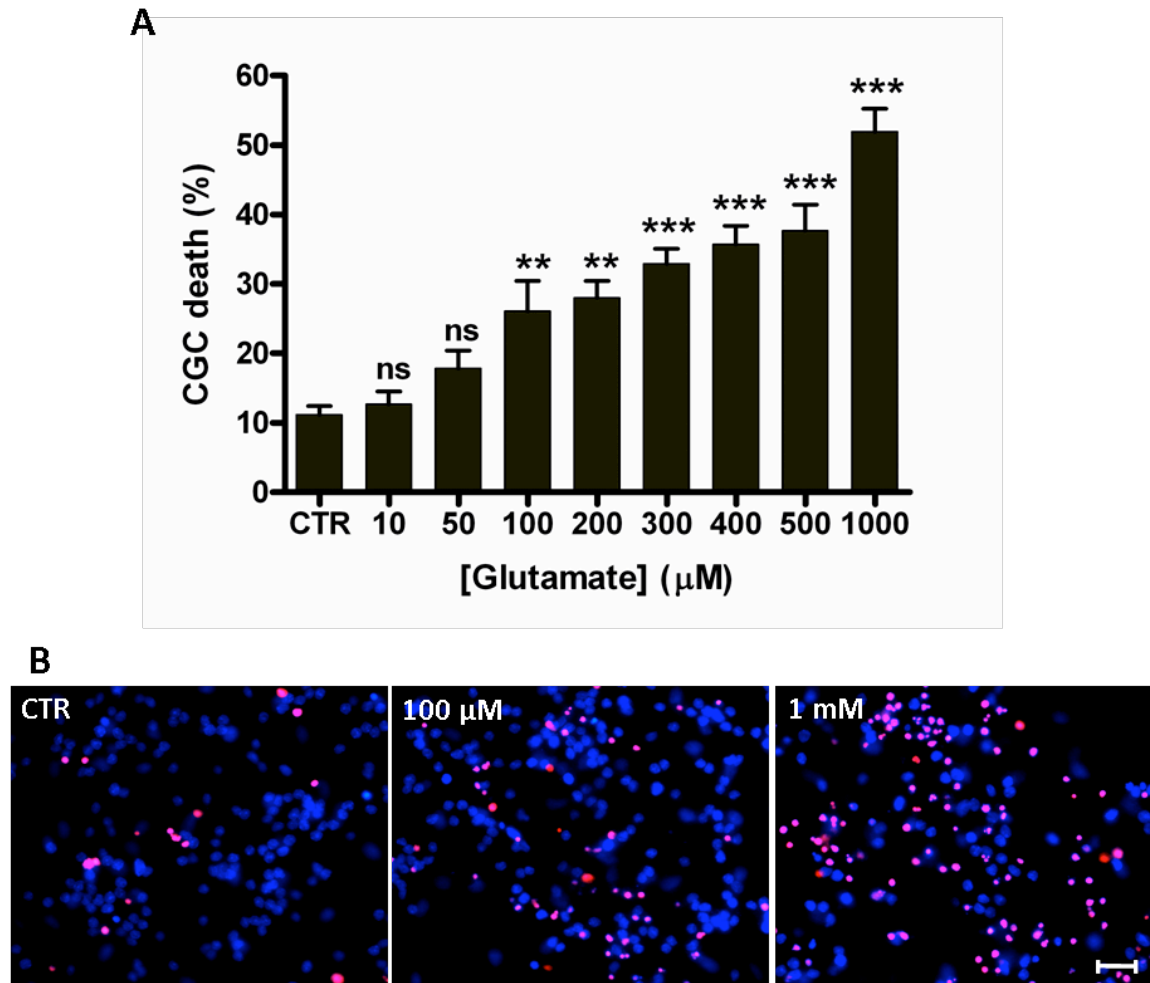
**Aiii**





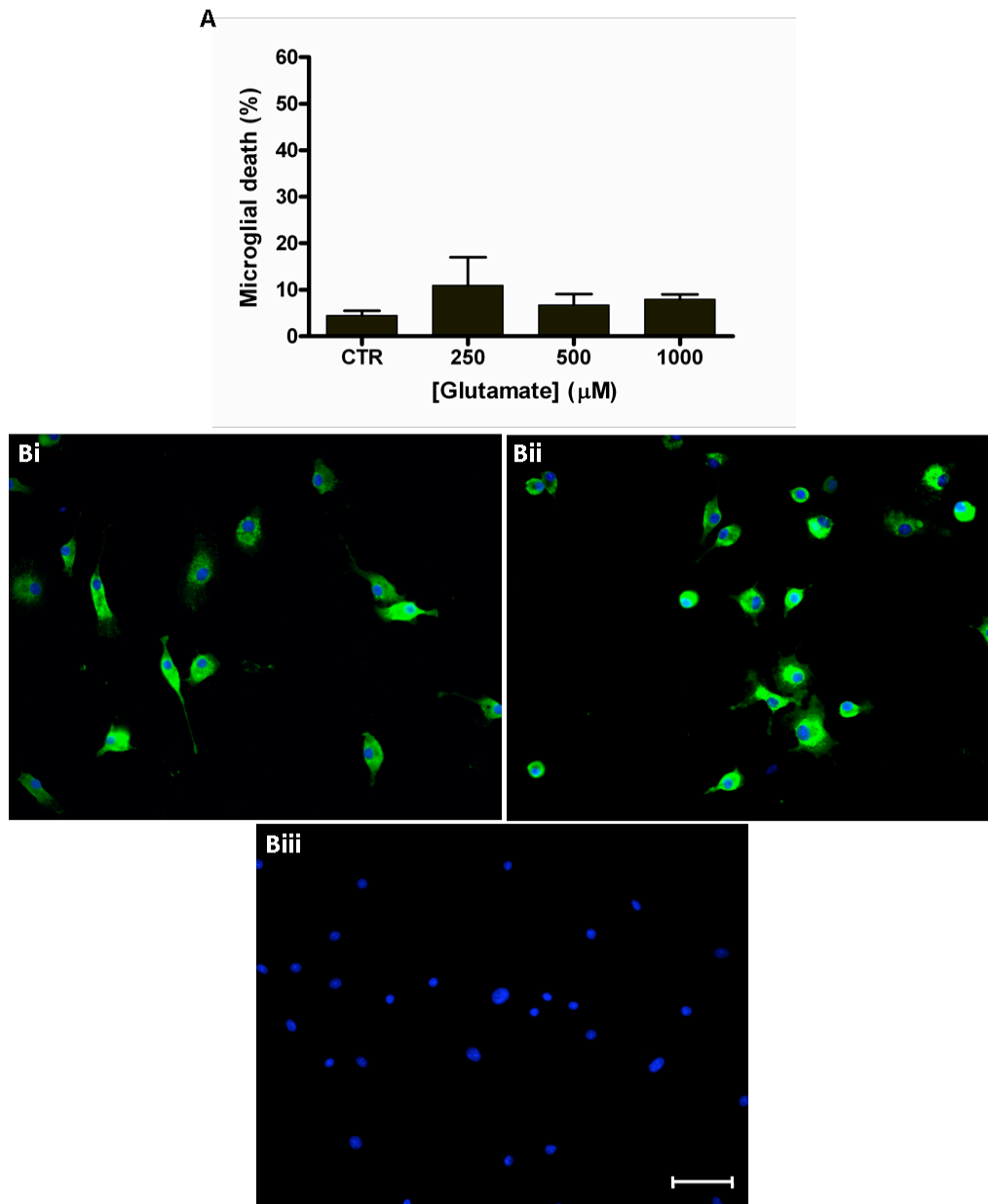
**Figure 4.5.1 Glutamate was toxic to OPCs and developing oligodendrocytes but not to mature oligodendrocytes**

Freshly made up glutamate was added to OPCs, 3 DIV developing oligodendrocytes and 6 DIV mature oligodendrocytes and the cells were incubated for 24 hours before cell death was assessed by live staining. Ai. Glutamate was significantly toxic to OPCs above 100  $\mu$ M. Aii. 3 DIV developing oligodendrocytes were vulnerable to glutamate above 500  $\mu$ M. Aiii. 6 DIV oligodendrocytes were not vulnerable to glutamate at any glutamate concentration used. B. Representative fluorescence microscopy images of OPCs (i), 3 DIV developing oligodendrocytes (ii) and 6 DIV oligodendrocytes (iii) stained with Hoechst (blue) and PI (red). Control, 100  $\mu$ M glutamate and 1 mM glutamate images are shown. Scale bar = 20  $\mu$ m. The values shown represent the mean  $\pm$  SEM of three experiments (four for OPCs) where three fields of each coverslip were counted. One way ANOVA with Tukey's post test was performed. Levels of significance were ns  $p>0.05$ , \*  $p<0.05$ , \*\*  $p<0.01$ , \*\*\*  $p<0.001$ .



**Figure 4.5.2 Glutamate was toxic to cerebellar granule cells**

Freshly made up glutamate was added to CGCs, an established model of excitotoxicity, and the cells were incubated for 24 hours before cell death was assessed by live staining. CGCs were vulnerable to glutamate in a concentration dependent manner and the plotted graph can be used to compare the effects of glutamate on an established model (CGCs), with glutamate toxicity to oligodendrocytes. Significant glutamate toxicity was observed from 100  $\mu$ M glutamate. B. Representative fluorescence microscopy images of CGCs stained with Hoechst (blue) and PI (red). Control, 100  $\mu$ M glutamate and 1 mM glutamate images are shown. Scale bar = 20  $\mu$ m. The values shown represent the mean  $\pm$  SEM of three experiments where three fields of each coverslip were counted. One way ANOVA with Tukey's post test was performed. Levels of significance were ns  $p > 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .



**Figure 4.5.3 Glutamate was not toxic to microglia**

A. Freshly made up glutamate was added to microglia and the cells were incubated for 24 hours before cell death was assessed by live staining. Glutamate was not toxic to microglia at any concentration used. B. Representative fluorescence microscopy images of Control (i), 1 mM Glutamate treated and negative control microglia (iii) incubated for 24 hours. Nuclei were stained blue with DAPI and the cytoplasm was stained green with anti-ED1 antibody. Glutamate treated microglia acquired an activated morphology with rounded, brightly stained cytoplasm. Scale bar = 20  $\mu$ m. Values shown represent the mean  $\pm$  SEM of three experiments where three fields of each coverslip were counted. One way ANOVA with Tukey's post test was performed. Level of significance was ns  $p > 0.05$ .

## 4.6 Discussion

In this chapter the effect of microglial activation with LPS on oligodendrocytes was investigated. It was shown that activated microglia were toxic to both OPCs and mature oligodendrocytes only when both cell populations were present. It was also shown that LPS induced OPC death was TNF- $\alpha$  and not NO mediated. mGluR manipulation did not seem able to modulate that toxicity. Mature oligodendrocytes were less vulnerable to activated microglia, but still co-culture of these two cell populations and LPS administration resulted in significant mature oligodendrocyte death. The properties of oligodendrocyte lineage cells do differ with the developmental stage of the cell population. Studying the effect of glutamate on oligodendrocytes further supported this. It was confirmed that indeed the vulnerability of the cells was dependent on their maturation stage, with mature oligodendrocytes being resistant to high concentrations of glutamate. The implications of these results and their clinical significance will be discussed.

### 4.6.1 Protective properties of microglial conditioned medium

In the literature it is strongly suggested that non activated microglial conditioned medium promotes OPC survival and maturation into oligodendrocytes (Pang et al., 2000; Nicholas et al., 2001; Miller et al., 2007). These results suggest that soluble factors released from microglia could have a supportive role for OPC survival and development. Insulin-like growth factor-2 (IGF-2) secreted in the medium of non-activated microglia was shown to reduce oligodendrocyte apoptosis *in vitro* (Nicholas et al., 2002) by recruiting phosphatidyl-3-inositol kinase and increasing NF- $\kappa$ B activation in OPCs (Nicholas et al., 2001). Untreated microglial conditioned medium did not evoke any significant changes to OPCs after 24 hours *in vitro* (Figure 4.3.1), although it is possible that longer incubation of the cells could be beneficial for their survival. Also, the basal death of the OPC cultures was very low (< 10%). When using the CG-4 cell line though, which had higher basal death than primary OPCs, BV-2 conditioned medium was protective to the CG-4s after incubation for 24 hours (Figure 4.3.2). In regards to mature oligodendrocytes, no protective or toxic

effect was attributed to non activated microglial conditioned medium (Figure 4.4.1). Non-activated microglial conditioned medium has also been implicated in neuronal survival *in vitro*. It has been shown that non-activated microglia can enhance neuronal survival up to 50% by acting on the mitogen-activated protein kinase and phosphatidylinositol-3-kinase/Akt signalling pathways (Morgan et al., 2004). The protective effects of microglial conditioned medium do not come as a surprise since microglia, as well as oligodendrocytes, neurones and astrocytes co-inhabit the brain and it is expected that in their 'relaxed' state they would be supportive to other cell populations.

Following LPS activation, microglia acquired an activated phenotype and expressed proteins indicative of an activated nature such as iNOS (Figure 4.2.2) and TNF- $\alpha$  (Figure 4.3.6). Activated microglial conditioned medium (MGCM) was found to be significantly protective to OPCs after a 24 hour incubation (Figure 4.3.1). This was also confirmed in the BV-2 and GC-4 cell lines (Figure 4.3.2). This finding came in contrast to a study showing that addition of LPS activated microglial conditioned medium to OPC cells, did not only abolish the protective effect of microglial conditioned medium, but induced OPC death (Pang et al., 2000). It has to be noted though, that in that study the microglia were activated with 1  $\mu$ g/ml LPS, a concentration tenfold higher than the one used here. It is the first time that a protective effect to OPCs has been attributed to LPS activated microglial conditioned medium. In a study complementary to this (Nicholas et al., 2002), IFN- $\gamma$  treated microglial conditioned medium increased the survival of CG-4 cells and oligodendrocytes. There were no significant changes to cell death levels when activated microglial conditioned medium was used to culture mature oligodendrocytes (Figure 4.4.1). The effects of conditioned medium on cells sometimes differ from the effects in co-cultures or *in vivo*, thus many researchers have resorted in working with co-cultures, where LPS activated microglia induce OPC death (discussed in 4.6.2). This model is also closer to the *in vivo* state of the cell populations.



#### **4.6.2 The TNF- $\alpha$ mediated pathway of LPS induced microglial toxicity to OPCs and developing oligodendrocytes**

Previous studies have shown that in co-culture, microglia activated with LPS induce OPC and developing oligodendrocyte death (Lehnardt et al., 2002; Li et al., 2008). This finding was confirmed and indeed incubation of microglial-OPC co-cultures with 0.1  $\mu\text{g/ml}$  LPS led to highly significant OPC death (Figure 4.3.4). The growth of microglia on cell inserts with 0.4  $\mu\text{m}$  pore size, allowing intercellular communication but compartmentalising the cells, again resulted in OPC death, but of lesser statistical significance than the death induced in co-culture. These results clearly indicated that the presence of microglia was necessary for LPS non cell autonomous OPC death. The attempts to elucidate the pathway of microglial activation that resulted into OPC death suggested TNF- $\alpha$  as the soluble factor released by activated microglia responsible for OPC toxicity. Inhibition of TNF- $\alpha$  synthesis with its synthesis inhibitor thalidomide, resulted in amelioration of the toxic effect of activated microglia (Figure 4.3.4). It has been shown that TNF- $\alpha$  treatment at a concentration of 100 ng/ml for 48 hours resulted in OPC toxicity that could be reversed by IGF-1 treatment (Pang et al., 2007). In a different study immature oligodendrocyte death in co-culture with microglia was caused by TNF- $\alpha$  and IFN $\gamma$  combination but only in the presence of astrocytes (Li et al., 2008). This group was unable to reproduce the effect of endogenous TNF- $\alpha$  by externally administering it. Additionally it was shown that OPC development to myelinating oligodendrocytes was sensitive to lower TNF- $\alpha$  concentrations than cell survival was (Cammer, 2000).

In mature oligodendrocytes it was shown that LPS activated microglia were toxic only in co-culture. This came in agreement with a study indicating mitochondrial depolarisation and mature oligodendrocyte cell death after microglial activation (Domercq et al., 2007) and in contrast to a study that produced evidence towards a protective role for LPS activated microglia in co-culture with mature oligodendrocytes (Miller et al., 2007). The mature oligodendrocyte death was not as extensive as in the OPC case but was still significant. Again the mechanism responsible for mature oligodendrocyte toxicity was TNF- $\alpha$  mediated (Figure 4.4.4), as also suggested by a much earlier study (Zajicek et al., 1992). The *in vitro* studies of

LPS non cell autonomous toxicity to mature oligodendrocytes are also supported by *in vivo* studies where substantial demyelination resulted from the focal inflammatory lesion caused by LPS injection directly into rat spinal cord (Felts et al., 2005; Marik et al., 2007).

It has been proposed that the mechanism by which TNF- $\alpha$  induces oligodendrocyte cell death only in co-culture and not via conditioned medium transferred from activated microglia to oligodendrocytes could involve cell contact and membrane bound microglial TNF- $\alpha$  which would bind to TNF receptors on oligodendrocytes (Zajicek et al., 1992). This theory though cannot explain the toxicity induced when cells are cultured separately in cell inserts that allow soluble factor exchange (Figure 4.3.3; Lehnardt et al., 2002; Li et al., 2005). There is also evidence suggesting that membrane bound TNF- $\alpha$  is the prime physiological activator of TNF- $\alpha$  receptor TNF-R2 (Grell et al., 1995). TNF-R2 is the TNF- $\alpha$  receptor implicated in neuroprotective cascades (Yang et al., 2002). Furthermore, in all experimental settings used the amount of TNF- $\alpha$  released was equal (Figure 4.3.6). In light of the fact that close proximity of the cells but not actual cell contact was required for microglial induced OPC toxicity, it is suggested that local, high concentration of TNF- $\alpha$ , possibly linked with the autocrine activation of microglia by TNF- $\alpha$  binding to microglial TNF-R1 and prompting release of additional TNF- $\alpha$  (Kuno et al., 2005) is required for toxicity to OPCs.

The involvement of NO and its by-products in the microglial mediated oligodendrocyte death was also examined. In the same study showing that immature oligodendrocyte death in co-culture with microglia was caused by TNF- $\alpha$  and IFN $\gamma$  combination only in the presence of astrocytes (Li et al., 2008), it was also suggested that in co-culture of pure microglia with immature oligodendrocytes the death mechanism switched to a NO and peroxynitrite mediated mechanism as previously reported (Li et al., 2005). An earlier study had also suggested that microglia kill immature oligodendrocytes via an NO mediated mechanism (Merrill et al., 1993). In mature oligodendrocytes it was shown that cells were specifically killed via peroxynitrite (Jack et al., 2007). In this study the use of the iNOS inhibitor AMT-HCl did not rescue OPCs from microglial mediated death (Figure 4.3.8 B). Even though the OPC cell cultures were highly enriched (92.8  $\pm$  1.29), with the majority of

contaminating cells being microglia, if the presence of astrocytes was responsible for protection against peroxynitrite and switch to the TNF- $\alpha$  mediated mechanism, this would probably represent the *in vivo* situation more convincingly. It has been suggested that the peroxynitrite mediated mechanism may dominate in demyelinating lesions with advanced cell death and astrocyte impairment (Li et al., 2008) .

The TNF- $\alpha$  receptors involved in OPC or mature oligodendrocyte death and the intracellular pathway involved were not investigated in this study, but there is convincing evidence that TNF- $\alpha$  acts on oligodendrocytes by binding to the TNF- $\alpha$  receptor TNF-R1. TNF-R1 is responsible for TNF- $\alpha$ 's apoptotic activity by triggering intracellular events involving a cascade that leads to activation of the transcription factors NF- $\kappa$ B and c-Jun and activation of caspase 8 (reviewed by Chen and Goeddel, 2002). It was shown that TNF- $\alpha$  release from microglia was coupled with apoptotic oligodendrocytes expressing TNF-R1 (Pang et al., 2007; Deng et al., 2008). TNF-R1 mRNA was shown to be expressed in both immature and mature oligodendrocytes, while TNF-R2 mRNA was not detected at any stage of maturation (Dopp et al., 1997). Interestingly, a later study showed that both TNF-R1 and TNF-R2 mRNA levels were upregulated in cuprizone induced demyelination (Arnett et al., 2001). Furthermore in TNF- $\alpha$  knockout mice cuprizone induced demyelination and apoptosis were delayed, and remyelination was promoted by OPC proliferation through TNF-R2 but not TNF-R1 binding of TNF- $\alpha$ .

#### **4.6.3 mGluR manipulation of microglial induced oligodendrocyte toxicity**

In the literature, there is information for a modulating role for mGluRs following microglial activation in studies where microglia were activated with LPS, amyloid  $\beta$  or chromogranin A peptide, compounds expressed in Alzheimer's plaques. Microglial activation caused microglial glutamate release, resulting in a feedback activation of group II mGluRs that fuelled microglial neurotoxicity (Taylor et al., 2002). It has been shown that group II mGluRs respond to much lower glutamate levels than group III mGluRs do (Pin and Duvoisin, 1995). Also, group III mGluR activation, possibly fuelled by higher glutamate concentrations, can be neuroprotective (Taylor et al., 2003).

Thus, group III mGluRs offer further targets for controlling microglial toxicity in neurodegenerative disease (reviewed by Pocock and Kettenmann, 2007). OPC death induced by microglial activation by LPS was not significantly modulated by the use of mGluR III agonists, or manipulation of other mGluRs (Figure 4.3.10). The TNF- $\alpha$  mediated detrimental effect of microglia to oligodendrocytes may be very robust to be modulated by mGluRs, it is thus interesting to investigate the results of microglial mGluR direct agonism or antagonism on oligodendrocytes. This is investigated and further discussed in Chapter 6.

#### **4.6.4 Glutamate excitotoxicity to oligodendrocytes depends on their maturation stage**

A number of reports have suggested that oligodendrocytes are very vulnerable to increased concentrations of glutamate (Oka et al., 1993; Yoshioka et al., 1995; Matute et al., 1997; McDonald et al., 1998; Li & Stys, 2000). Here it was observed that glutamate was significantly toxic to OPCs and developing oligodendrocytes (Figure 4.5.1 A). Surprisingly, it was observed that even high, physiologically non-relevant concentrations of glutamate were unable to induce cell death of 6 DIV oligodendrocytes (Figure 4.5.1). These results were compared to the effect of glutamate to CGCs where significant, concentration dependent excitotoxicity was observed within 24 hours (Figure 4.5.2). On the other hand, microglia did not die in response to high glutamate concentrations (Figure 4.5.3), although 1mM glutamate rendered them unable to respond to LPS and exhibit toxicity to mature oligodendrocytes (Figure 4.4.6).

On closer inspection of the literature, it was noticed that in most studies where oligodendrocyte toxicity was mentioned, the cells under investigation were pre-myelinating cells that had not been kept in culture long enough and were not proven to express myelin proteins (Oka et al., 1993; Matute et al., 1997; Deng et al., 2006; DeSilva et al., 2009). The CG4 cell line was also used and differentiated in oligodendrocyte 'like' cells (Yoshioka et al., 1995). It has been reported though that CG-4 cells do not completely differentiate into mature oligodendrocytes (Itoh et al., 2000). Interestingly, in a study where AMPA induced oligodendrocyte death, the

cells were left to mature for long periods of time but in the presence of an astrocyte layer (McDonald et al., 1998). A more recent study of whole spinal cord sections showed conduction impairment and damage to the white matter after glutamate administration (Li & Stys, 2000). Two *in vitro* studies of mature oligodendrocytes expressing myelin proteins have indeed shown insensitivity to the specific glutamate receptor kainate (KA) in rat (Rosenberg et al., 2003) or human (Wosik et al., 2004) oligodendrocytes. It is possible that mature oligodendrocytes *in vitro* do not respond to glutamate, but maybe when astrocytes are present there is a shift towards a toxic cascade, mediated by glutamate challenged astrocytes.

The excitotoxic effect of glutamate on oligodendrocytes would be expected to depend on their expression of ionotropic glutamate receptors, which is an area of extensive research. In support of the findings of the current study, AMPA receptor subunits GluR2, GluR3 and GluR4 were expressed in developing oligodendrocytes, but in mature oligodendrocytes only GluR2 remained (Itoh et al., 2002). Sole expression of GluR2 has been shown not to evoke a  $\text{Ca}^{2+}$  current (Hollmann et al., 1991; Itoh et al., 2002). Kainate receptor subunits were also downregulated in mature oligodendrocytes (Rosenberg et al., 2003). It was believed that oligodendrocytes did not possess NMDA receptors, but recently white matter oligodendrocytes of all developmental stages were shown to express functional NMDA receptors (Káradóttir et al., 2005). In mature oligodendrocytes these receptors are believed to be expressed in the cell processes in the compact myelin around axons and it was suggested that they might control axon-oligodendrocyte interactions, being too deep within the myelin to ever be exposed to glutamate (reviewed by Káradóttir and Attwell, 2007).

#### **4.6.5 Conclusion**

The findings that LPS activated microglia were toxic to oligodendrocytes via  $\text{TNF-}\alpha$  secretion have clinical significance in respect to MS, which is highlighted by the fact that microglial activation is implicated in MS (see 4.4.1), but also by the fact that infectious pathogens have been proposed as potential environmental risk factors for MS, such as EBV, human herpes virus 6, MS-associated human endogenous

retroviruses and the gram negative bacterial pathogen *Chlamydia pneumoniae* (reviewed by Giovannoni et al., 2006). Microglial activation leading to developing oligodendrocyte death could be a factor contributing to remyelination failure. A role for activated microglia in mature oligodendrocyte death is also delineated.

The clinical significance of this chapter's finding that glutamate is toxic predominantly to developing oligodendrocytes is rendered important by studies that have confirmed elevated levels of glutamate in MS. In the CSF of MS patients, glutamate levels were found elevated during relapses or in patients with severe neurological defect (Stover et al., 1997; Barkhatova et al., 1998). Glutaminase, the enzyme that converts glutamine to glutamate, and glutamate transporter elevated levels were correlated with axonal damage in MS patient tissue (Werner et al., 2001). Elevated glutamate could prevent remyelination in lesions by directly injuring developing oligodendrocytes, or by indirectly contributing to mature oligodendrocyte death by its action on microglia and astrocytes. Thus, regulation of glutamate signalling in MS lesions could be beneficial to patients and promote remyelination.

## **5. INVESTIGATION OF OLIGODENDROCYTE AND NEURONAL VULNERABILITY TO BLOOD - BORNE PROTEINS**

## 5.1 Introduction

Following BBB disturbance, a typical event in MS (Kermode et al., 1990; Gay & Esiri, 1991), neurodegenerative disease (Alafuzoff et al., 1987; Claudio, 1996; Fiala et al., 2002) and ischemia (Hornig et al., 1983), soluble blood-borne proteins (BBPs) that are physiologically excluded from the CNS leak into the brain (see 1.4.2). Elevated levels of BBPs in the brain could contribute to MS pathology by acting upon microglia and affecting oligodendrocyte survival. Thus, in this chapter the direct effect of the BBPs fraction V albumin, fibrinogen and fibrin, as well as the effect of BBP activated microglia on the viability of primary oligodendrocytes was studied. Fraction V albumin, fibrinogen and fibrin were selected for this study based on published work showing that they can activate microglia *in vitro* (see below).

### 5.1.1 FV albumin (FV)

Albumin is a 66 kDa protein constituting approximately 50% of all plasma proteins. It binds fatty acids released into the blood, contributes to the colloid osmotic blood pressure, buffers pH in the blood and controls vascular permeability to water. It is present in blood at a concentration of 35-50 mg/ml. Partial purification results in fraction V serum albumin in which the albumin contains bound fatty acids and coagulation factors (reviewed by Nadal et al. 2001). Fraction V albumin (FV) was used in this study as a model of physiological albumin, as it represents albumin *in vitro* better than purified albumin where only the protein part of the complex is preserved. Albumin is normally excluded from the brain (concentration in the brain is 35-50 µg/ml) but following BBB breakdown, albumin can enter the brain (Salahuddin et al., 1988; Sokrab et al., 1988; Butter et al., 1991; Kevil et al., 1998).

Elevated albumin concentrations in the CNS can exert diverse effects on brain cells. Astrocytes respond to albumin by calcium uptake and proliferation, events resembling astrocyte response to CNS damage (Nadal et al., 1996). In microglia, albumin can act as a signalling molecule, inducing an increase in cytoplasmic calcium, mediated via Src tyrosine kinases and phospholipase C, and leading to calcium-dependent proliferation (Hooper et al., 2005). It was also shown that microglial



activation with lipid free albumin resulted in superoxide production *in vitro* (Si et al., 1997) and it was recently shown that albumin or FV induced iNOS expression in microglia via a MAPK dependent pathway (Hooper et al., 2009). FV also induced glutamate and TNF- $\alpha$  release, glutathione production and was implicated in neurotoxic cascades following microglial activation (Hooper et al., 2009). Here the direct effect of FV, as well as the effect of FV activated microglia on OPC and oligodendrocyte toxicity was studied.

### 5.1.2 Fibrinogen (FG) and fibrin (FN)

Fibrinogen (FG) is a 340 KDa soluble blood plasma glycoprotein that comprises of three pairs of non-identical polypeptide chains (A $\alpha$ , B $\beta$  and  $\gamma$  chains) linked with disulphide bonds. It circulates in the blood plasma at 1.5 - 4.5 mg/ml (reviewed by Kamath & Lip, 2003). FG is involved in blood coagulation where it is cleaved by the serine protease thrombin into cleavage products with polymerization sites that subsequently form an insoluble clot polymer, fibrin (FN) (Lord, 2007). FN networks form a 3D mesh of compact straight or rod-like elements. In disease, FN networks demonstrate abnormal architecture, for example in atherothrombosis, FN comprises of stiffer polymers made of many short fibers (Collet et al., 2006). Following BBB disturbance FN deposits as an insoluble mesh into the brain and may damage it by preventing neurite outgrowth (Akassoglou et al., 2000). FG and FN leak into the brain after BBB disturbance in MS (Claudio et al., 1995; Wakefield et al., 1994; Gay et al., 1997; Gveric et al., 2001), ischemia (Schoots et al., 2003) and late stage Alzheimer's (Thomas et al., 1996; Merkle et al., 1996; Melchor et al., 2003).

FG and FN are also implicated in macrophage activation (Perez & Roman, 1995) microglial activation and proliferation (Adams et al., 2007). Moreover, a FG-derived peptide known to inhibit the Mac-1 integrin receptor on microglia ( $\gamma^{377-395}$  peptide), blocked microglial activation *in vitro*. *In vivo* studies using either the inhibitory  $\gamma^{377-395}$  peptide or mice expressing a mutant form of FG lacking the Mac-1 binding motif (FG knock-in mice) showed that targeting the inflammatory but not the procoagulant properties of FG was sufficient to suppress microglial activation and inflammation. FG- $\gamma^{390-396A}$  knock-in mice or pharmacological impediment of FG–Mac-1 interaction

through intranasal delivery of the inhibitory  $\gamma^{377-395}$  peptide attenuated microglia activation and suppressed relapsing paralysis in EAE mice (Adams et al., 2007). The presence of FG and FN in MS tissue may exacerbate microglial and macrophage activation, so the effect of FG and FN on microglial activation and oligodendrocyte lineage cell viability was assessed.

### 5.1.3 Rho-ROCK pathway inhibition

ROCK (Rho-associated coiled coil-containing protein kinase) is a Rho-GTP binding kinase, which exists in two isoforms, ROCK I and ROCK II, which is preferentially expressed in the brain (Nakagawa et al., 1996). In its active form ROCK activates numerous downstream effectors by phosphorylating proteins such as myosin light chain (Leung et al., 1996), LIM kinases 1 (Ohashi et al., 2000) and 2 (Sumi et al., 2001), tau, MAP2 (Amano et al., 2003), GFAP (Kosako et al., 1997), and neurofilaments (Hashimoto et al., 1998), that depolymerise upon phosphorylation. ROCK regulates actin-myosin contractivity and is thus implicated in cytoskeleton rearrangement and cell process retraction (reviewed by Mueller et al., 2005).

ROCK inhibition has been proposed as a feasible neurite outgrowth enhancing strategy. In spinal cord injury, Rho-ROCK stimulation by the trimeric NgR/p75<sup>NTR</sup>/LINGO-1 receptor complexes caused neurite growth inhibition (Mi et al., 2004). Neurite outgrowth was promoted when ROCK was pharmacologically inhibited (Lingor et al., 2007). In Alzheimer's disease inhibition of the Rho-ROCK pathway resulted in reduction of the amyloid fibril peptide A $\beta$ 42 levels (Zhou et al., 2003). There is also evidence that ROCK inhibition is neuroprotective against glutamate-related excitotoxicity (Kitaoka et al., 2004). In MS ROCK was implicated in trans-endothelial migration of leukocytes, which was prevented by inhibiting ROCK with its specific inhibitor Y-27632 (Honing et al., 2004). Simvastatin, a lipophilic statin that has Rho-ROCK inhibition mediated anti-inflammatory properties is being evaluated as a potential MS therapy (Paintlia et al., 2005; Miron et al., 2007). ROCK was also identified as a key factor in coordinating Schwann cell myelination by regulating myosin phosphorylation (Melendez-Vasquez et al., 2004).

ROCK involvement in myelination, oligodendroglial survival regulation (Miron et al., 2007) and neurite outgrowth (Lingor et al., 2007) raise questions about the involvement of the Rho-ROCK pathway in reducing OPC or neuronal survival. In this study ROCK inhibition with Y-27632 was aimed at attenuating microglial activation and cell toxicity induced by direct addition of BBPs on oligodendrocytes.

## 5.2 Microglial activation by blood-borne proteins

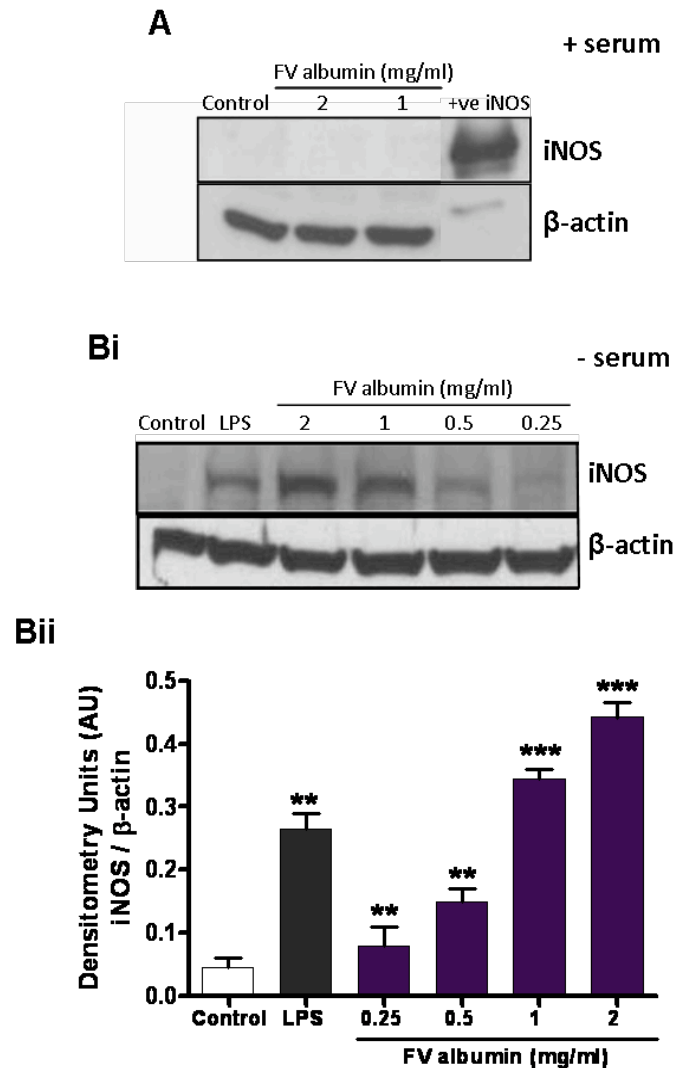
To assess iNOS expression in BV-2 cells following activation with FV, BV-2s were incubated with FV in serum containing medium for 24 hours. No iNOS expression was detected with denaturing western blotting (Figure 5.2.1 A). Cells were then plated in serum containing medium that was replaced with serum free medium for at least two hours before cell activation. Activation in serum free medium resulted in iNOS expression in a dose dependent way (Figure 5.2.1 B). Primary microglia were also activated with FV in serum free medium and iNOS expression was detected by immunostaining (Figure 5.2.2 A) and western blotting (Figure 5.2.2 B).

BV-2 cells and primary microglia were also activated with the BBPs FG and FN. Following initial observations that only FN but not FG could induce iNOS expression in serum containing medium (Dr Emma East, unpublished data), and having already been successful at inducing iNOS expression with FV when culturing microglia in serum free medium, the experiments with FG and FN were performed in serum free conditions. BV-2 cells were activated with 2.5 mg/ml FG or 1 mg/ml FN for 24 hours and iNOS expression similar to that induced with 1 µg/ml LPS was detected with denaturing western blotting (Figure 5.2.3). Primary microglia were also activated with FG and FN in serum free medium and iNOS expression was detected by immunostaining (Figure 5.2.4 A) and western blotting (Figure 5.2.4 B). iNOS expression of FG and FN activated microglia was comparable to iNOS expression of LPS activated microglia (Figure 5.2.4).

Finally, TNF-α release from BBP activated microglia was assessed. TNF-α was released following microglial activation with LPS (discussed in Chapter 4, Figure 4.3.6), so the TNF-α release from microglia following BBP activation was also measured using a TNF-α ELISA kit (see 2.8). FV, FG and FN activated microglia for 24 hours all released TNF-α (Figure 5.2.5 A). Because the interactions between BBP activated microglia and oligodendrocytes was the aim of this study, TNF-α release in the presence of OPCs or 6 DIV oligodendrocytes was measured. Microglial TNF-α release was measured when microglia were cultured on 0.4 µm inserts above OPCs or 6 DIV oligodendrocytes for 24 hours, or when microglia were cultured in contact with OPCs or 6 DIV oligodendrocytes, in MG-OPC or MG-6 DIV oligodendrocyte co-

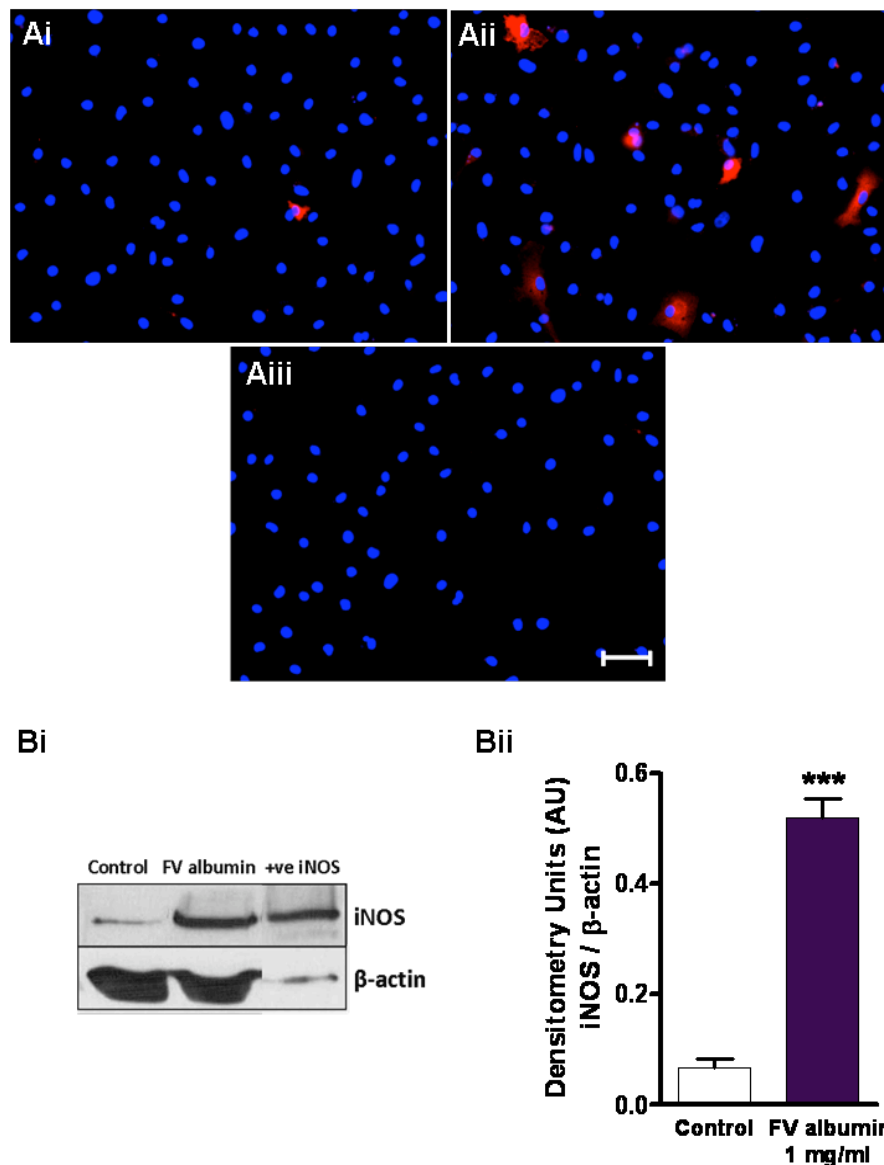
cultures (Figure 5.2.5 B, C). In all cases TNF- $\alpha$  was released. In co-culture with OPCs, FN activated microglia released less TNF- $\alpha$  than the amount released from microglia alone, or microglia cultured on inserts (Figure 5.2.6 Aiii). This may be due to microglial apoptosis following FN activation and internalisation of FN present in the medium, resulting in fewer microglia in co-culture (see 5.3.3). When microglia were cultured above 6 DIV oligodendrocytes on inserts, or with 6 DIV oligodendrocytes in contact TNF- $\alpha$  released following activation with FV, FG or FN was lower than that released when microglia were cultured alone (Figure 5.2.5). FG activation of microglia consistently resulted in release of lower levels of TNF- $\alpha$  compared with TNF- $\alpha$  released following activation with FV or FN. It is possible that after 6 DIV *in vitro* the tissue culture environment was more toxic, resulting in microglial apoptosis in the inserts, thus less microglia were able to release TNF- $\alpha$ . In co-culture experiments, where the cells were cultured in contact with 6 DIV oligodendrocytes, lower levels of TNF- $\alpha$  could be attributed to the fact that in co-culture the number of microglia present in a well is smaller since it is only a percentage of the total cell population.

Collectively, it was shown that following LPS, FV, FG or FN activation, microglia expressed iNOS and released TNF- $\alpha$ , both indicative of a reactive microglial nature. The effect of this microglial activation, as well as the direct effect of the BBPs used in this study on oligodendrocytes was subsequently investigated.



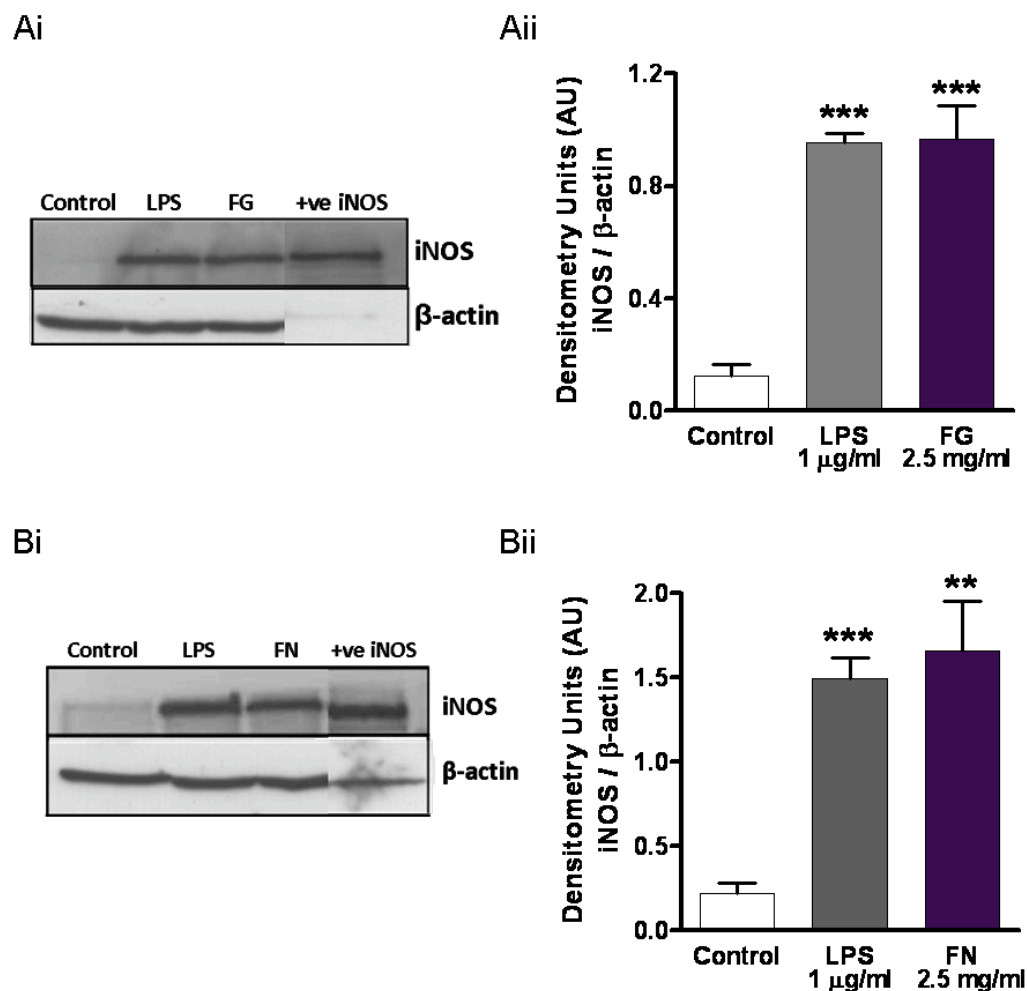
**Figure 5.2.1 Presence of serum blocked FV albumin induced iNOS increase in BV-2 cells**

A. Representative western blot of iNOS expression of 1 day *in vitro* BV-2s after 24 hour activation with FV albumin (FV) in serum containing medium. No iNOS was detected B. Representative western blot (i) and densitometric analysis (ii) of iNOS expression of 1 day *in vitro* BV-2s after 24 hour activation with FV in serum free medium. FV induced iNOS expression in serum free medium in a dose dependent manner and 1 mg/ml FV produced a similar amount of iNOS to 1 µg/ml LPS. β-actin was used as a loading control. The experiments were repeated in duplicate. Student's t-tests (for control vs. LPS) and one way ANOVA with Tukey's post test (for control vs. FV) were performed. Levels of significance were ns  $p>0.05$ , \*\*  $p<0.01$ , \*\*\*  $p<0.001$ .



**Figure 5.2.2 FV albumin induced iNOS expression in primary microglia**

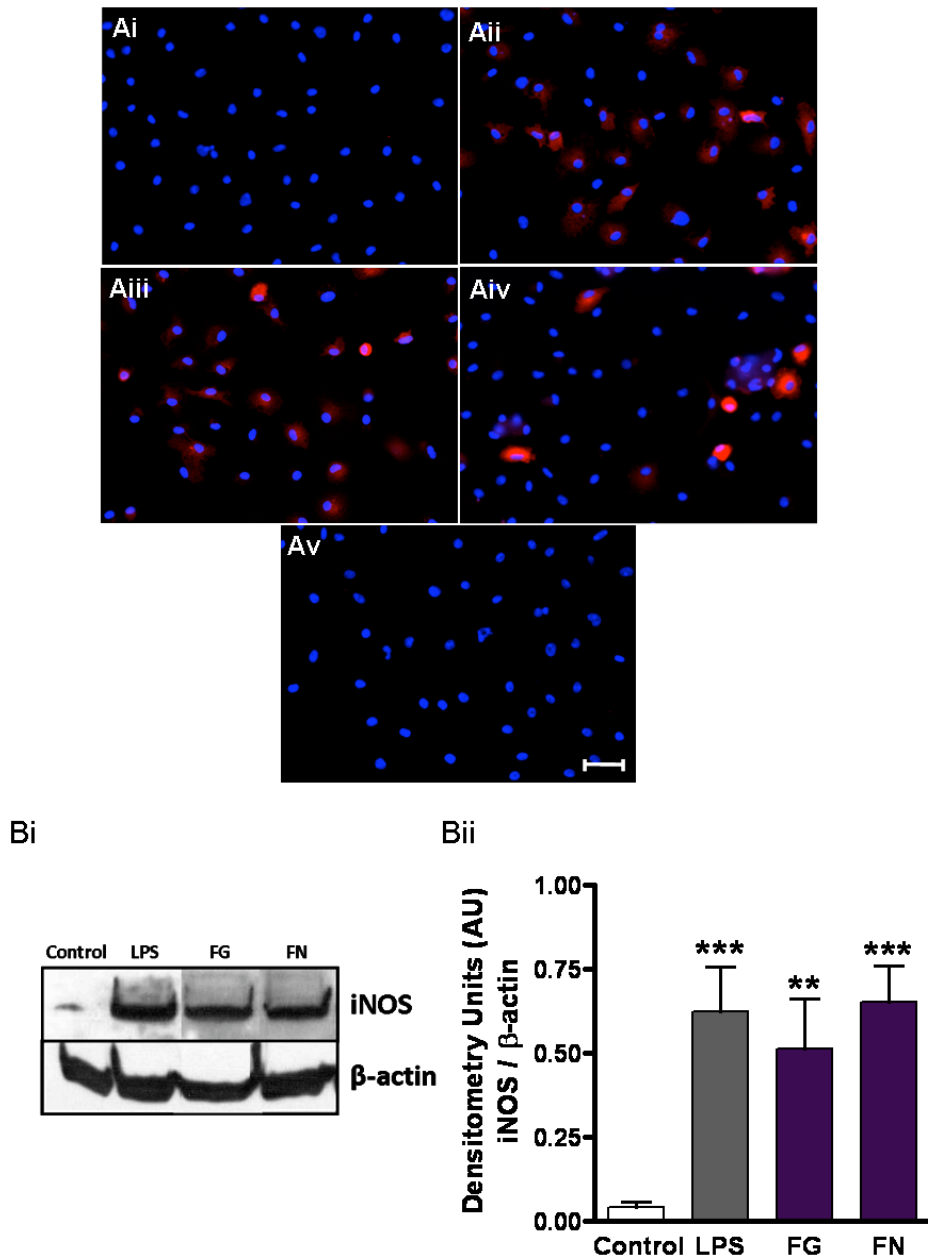
A. Representative fluorescence microscopy images of (i) control, (ii) 1 mg/ml FV and (iii) negative control (primary antibody omitted) treated fixed primary microglia in serum free culture medium, following 24 hour incubation. Nuclei were stained blue with DAPI and the cytoplasm was stained red with anti-iNOS antibody. Scale bar = 20  $\mu$ m. B. Representative western blot (i) and densitometric analysis (ii) of iNOS expression of 1 day *in vitro* primary microglia after 24 hour activation with FV albumin (FV) in serum free medium. 1 mg/ml FV induced iNOS expression. Commercially available mouse macrophage LPS/IFN $\gamma$  cell lysate (BD Transduction 611473) was used as positive control.  $\beta$ -actin was used as a loading control. The experiments were repeated in triplicate and student's t-test was performed to compare the two groups. Level of significance was \*\*\*  $p < 0.001$ .



**Figure 5.2.3 Fibrinogen and fibrin induced iNOS expression in BV-2 cells**

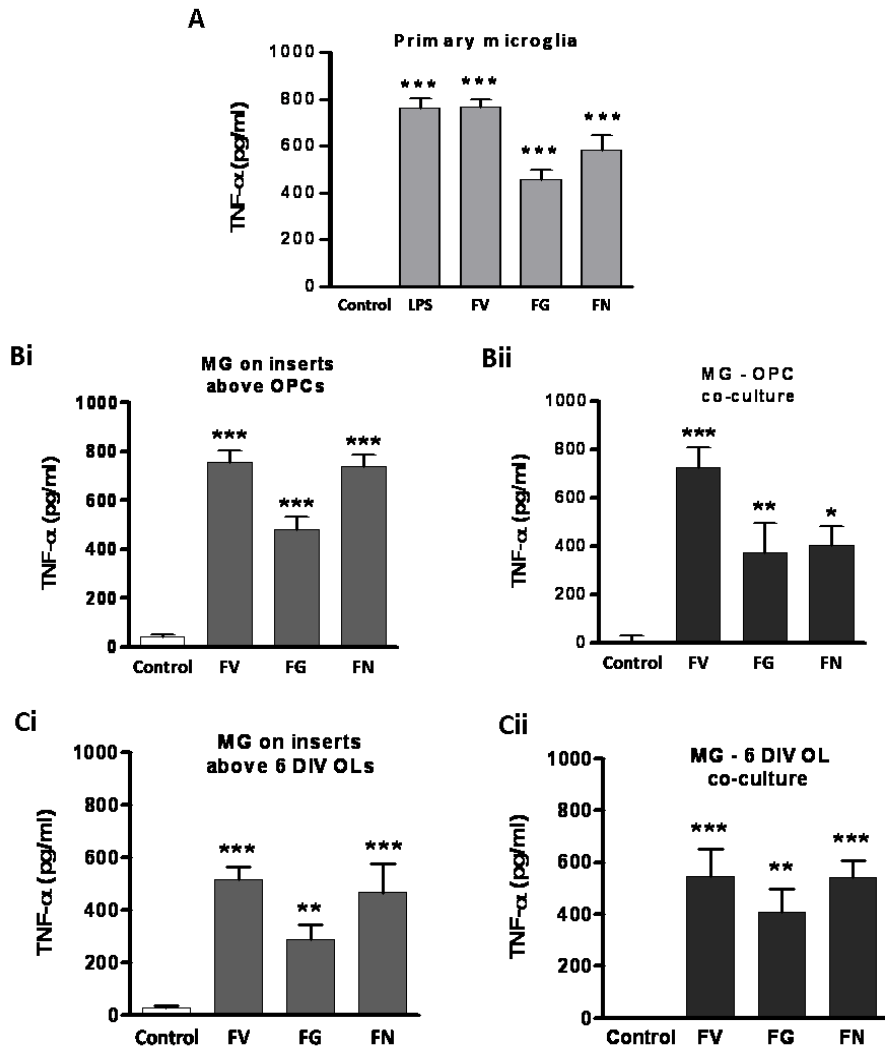
A. Representative western blot (i) and densitometric analysis (ii) of iNOS expression of 1 day *in vitro* BV-2s after 24 hour activation with 1  $\mu$ g/ml LPS or 2.5 mg/ml fibrinogen (FG) in serum free medium. 2.5 mg/ml FG induced similar levels of iNOS as 1  $\mu$ g/ml LPS. B. Representative western blot (i) and densitometric analysis (ii) of iNOS expression of 1 day *in vitro* BV-2s after 24 hour activation with 1  $\mu$ g/ml LPS or 1 mg/ml fibrin (FN) in serum free medium. 1 mg/ml FN induced similar levels of iNOS as 1  $\mu$ g/ml LPS. Commercially available mouse macrophage LPS/IFN $\gamma$  cell lysate (BD Transduction 611473) was used as positive control.  $\beta$ -actin was used as a loading control. The experiments were repeated in triplicate and student's t-tests were performed to compare the two groups with the control. Levels of significance were \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .





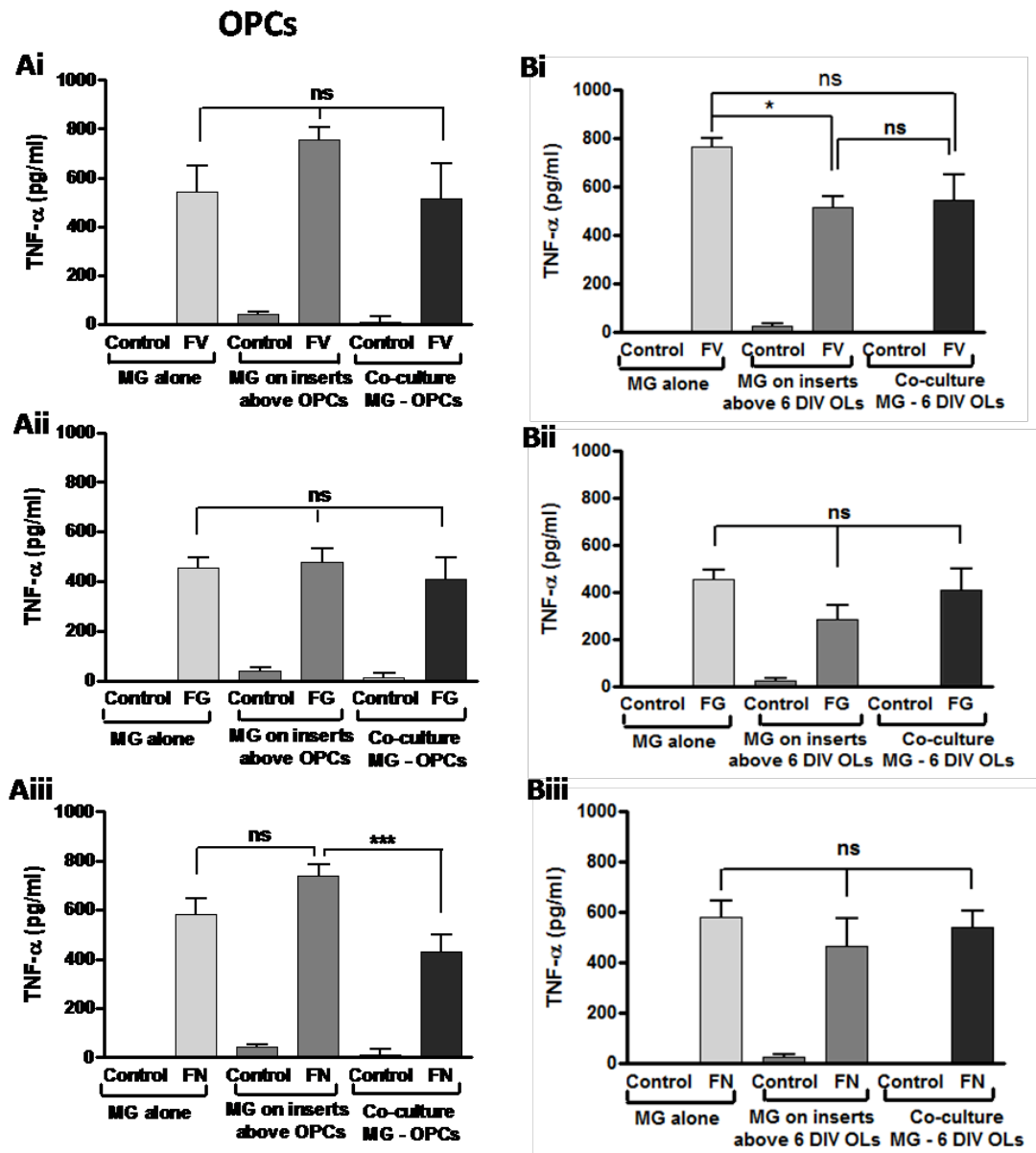
**Figure 5.2.4 Fibrinogen and fibrin induced iNOS expression in primary microglia**

A. Representative fluorescence microscopy images of (i) control, (ii) 1  $\mu$ g/ml LPS, (iii) 2.5 mg/ml FG, (iv) 1 mg/ml FN and (v) negative control (primary antibody omitted) treated fixed primary microglia in serum free culture medium, following 24 hour incubation. Scale bar = 20  $\mu$ m. Nuclei were stained blue with DAPI and the cytoplasm was stained red with anti-iNOS antibody. B. Representative western blot (i) and densitometric analysis (ii) of iNOS expression of 1 day *in vitro* primary microglia following 24 hour activation with 1  $\mu$ g/ml LPS, 2.5 mg/ml FG (ii) or 1 mg/ml FN in serum free medium.  $\beta$ -actin was used as a loading control. The experiments were repeated in triplicate and student's t-tests were performed to compare all groups with the control. Levels of significance were \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .



**Figure 5.2.5 FV albumin, fibrinogen and fibrin induced TNF-α release from microglia in the presence of oligodendrocyte lineage cells**

Primary microglia were cultured alone, in inserts above OPCs or above 6 DIV oligodendrocytes, or in co-culture with OPCs or 6 DIV oligodendrocytes. Microglia were activated for 24 hours with 1 mg/ml FV, 2.5 mg/ml FG, or 1 mg/ml FN. A. FV activated microglia released TNF-α as potently as LPS, while FG and FN activated microglia released lower but still significant levels of TNF-α. B. Microglia were cultured above OPCs on inserts (i), or with OPCs in contact (ii). TNF-α was released and the amount released following activation with FV, FG or FN was similar to that released when microglia were cultured alone. In co-culture FN activated microglia released less TNF-α. This may be due to microglial apoptosis resulting in fewer microglia in co-culture, following FN activation and internalisation of FN present in the medium (see 5.3.3). C. Microglia were cultured above 6 DIV oligodendrocytes on inserts (i), or with 6 DIV oligodendrocytes in contact (ii). TNF-α released following activation with FV, FG or FN was lower than that released when microglia were cultured alone. FG activation consistently resulted in lower levels of TNF-α release than FV or FN activation of microglia. All treated groups were compared with the control using one way ANOVA with Tukey's post test. Levels of significance were \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .



**Figure 5.2.6 Comparison of TNF- $\alpha$  release from microglia when cultured alone, on inserts above oligodendrocytes or in contact co-culture with oligodendrocytes**

Primary microglia were cultured in inserts above OPCs or in co-culture with OPCs (A) and in inserts above 6 DIV oligodendrocytes or in co-culture with 6 DIV oligodendrocytes (B). Microglia were activated for 24 hours with 1 mg/ml FV, 2.5 mg/ml FG, or 1 mg/ml FN. Comparisons between different experimental conditions following microglial activation with BBPs were made for OPCs or 6 DIV oligodendrocytes. In co-culture FN activated microglia released less TNF- $\alpha$  (Aiii). This may be due to microglial apoptosis resulting in fewer microglia in co-culture, following FN activation and internalisation of FN present in the medium (see 5.3.3). (Bi). One way ANOVA with the Bonferroni correction was performed to compare all groups. Levels of significance were ns  $p > 0.05$ , \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

## 5.3 Effect of BBPs on OPCs

### 5.3.1 Effect of FV albumin on OPCs

OPC cultures alone were incubated with direct administration of 1 mg/ml FV for 24 hours. FV was significantly toxic to OPCs as assessed by live-dead staining (Figure 5.3.1.1 A). An attempt to prevent FV-induced OPC death was made by pre-incubating the cells for 24 hours with 50  $\mu$ M of the ROCK inhibitor Y-27632 (Maekawa et al., 1999), followed by 24 hours incubation with FV. The rationale for inhibiting ROCK to prevent FV-induced OPC death was based on reports for ROCK involvement in myelination and regulation of oligodendroglial survival (Miron et al., 2007), as well as on the finding that ROCK blockade prevented FN induced death (see Figure 5.3.3 A). Y-27632 binds to the catalytic site of the kinase and competitively antagonises ATP (Ishizaki et al., 2000). The 24 hour pre-incubation would allow for Y-27632 to be taken up by the microglia and bind to the catalytic site of the kinase. Y-27632 was unable to prevent FV induced death (Figure 5.3.1.1 A). It should be noted though that 48 hour incubation with Y-27632 was significantly toxic to OPCs.

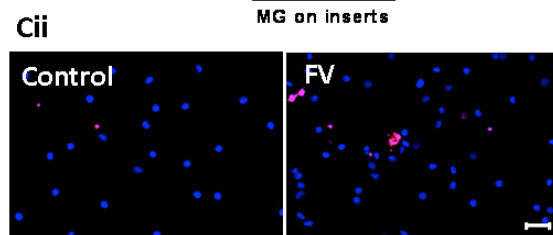
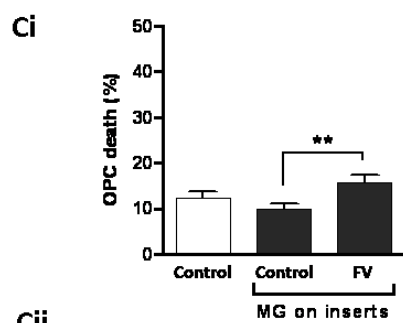
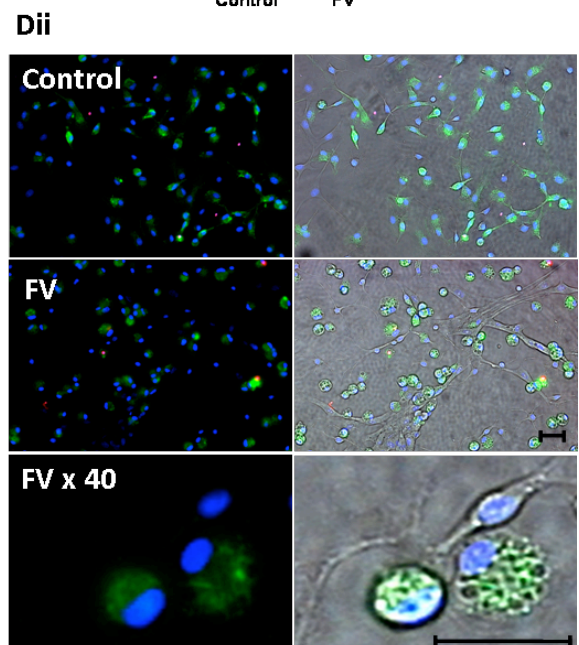
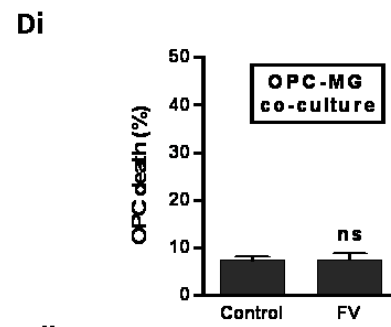
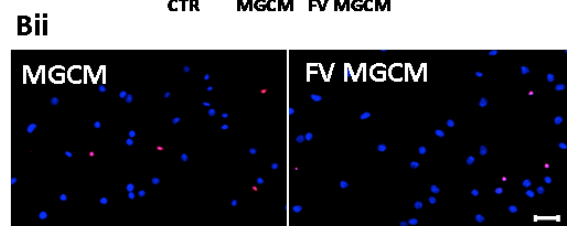
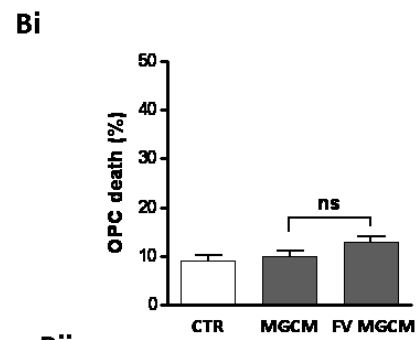
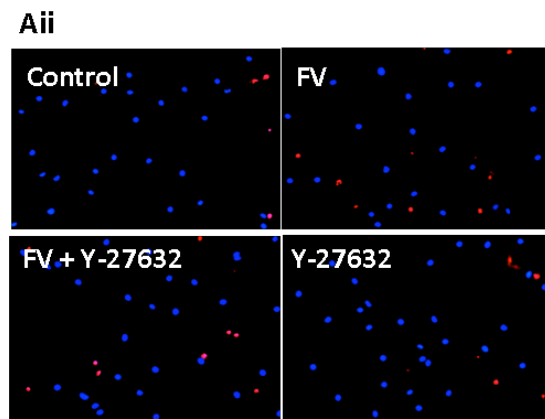
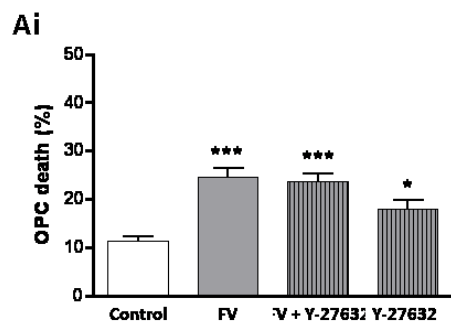
To study the effect of FV activated microglia on OPCs, microglial conditioned medium (MGCM) activated with 1 mg/ml FV (potent microglial activator, see Figure 5.2.2) was applied to OPCs at a 1:1 ratio to OPC culture medium for 24 hours. FV activated MGCM was not toxic to OPCs after 24 hour treatment (Figure 5.3.1.1 B). When microglia were cultured above OPCs in 0.4  $\mu$ m pore inserts that only allowed soluble factor transport, FV induced OPC death although not at the same extent as when applied directly on the OPCs in the absence of microglia in the culture system (Figure 5.3.1.1 C). Interestingly though, when the two cell populations were cultured together and were in contact (hereafter referred to as MG-OPC co-culture), administration of FV did not induce OPC death (Figure 5.3.1.1 D). This shows that the presence of microglia is able to protect OPCs from FV induced death especially when the cells are in close proximity (co-culture).

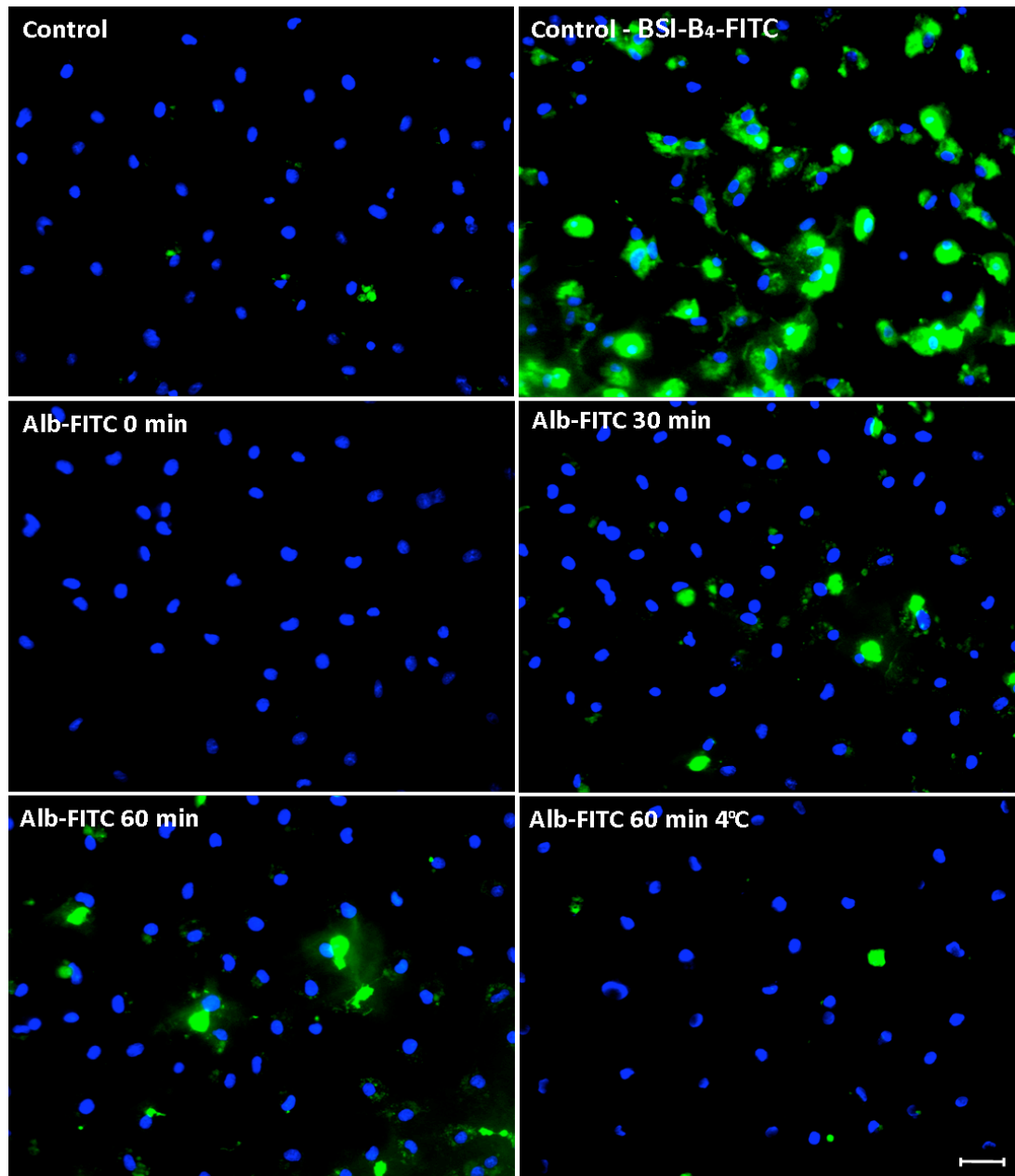
Microglia exhibited a highly phagocytic phenotype in the presence of FV (Figure 5.3.1.1 D), so it can be speculated that microglia protect OPCs by taking up the FV

present in the culture medium. Indeed, in an experiment to assess the phagocytic ability of microglia in response to albumin, primary microglia were incubated with FV albumin for 2 hours to prime internalisation, followed by incubation with 1 mg/ml FITC conjugated albumin (Alb-FITC; green) for 0, 30, or 60 minutes at 37°C to assess albumin internalisation by live staining. After 30 or 60 minutes of incubation Alb-FITC was internalised by microglia and punctate staining indicative of vesicular location of Alb-FITC was observed (Figure 5.3.1.2). Cells were also incubated with Alb-FITC for 60 min at 4°C to prevent internalisation and to ensure that the observed fluorescence was not due to binding (Tabernero et al., 1999; Hooper, 2003; Pinteaux-Jones, 2007). Some cells were stained with FITC conjugated isolectin BSI-B<sub>4</sub> to selectively stain microglia, view their morphology and confirm cell culture purity (Figure 5.3.1.2).

### Figure 5.3.1.1 Activated microglia protected OPCs from direct FV albumin toxicity

A. OPCs cultured alone were incubated with 1 mg/ml FV albumin for 24 hours or preincubated for 24 hours with 50 mM of the ROCK inhibitor Y-27632 followed by FV for another 24 hours. Cells were also treated with Y-27632 alone for 48 hours to assess Y-27632 toxicity. FV induced OPC death. ROCK inhibition was unable to prevent it. The ROCK inhibitor Y-27632 was significantly toxic to the cells. B. OPCs cultured alone were incubated with 50% MGCM for 24 hours. Microglia were left untreated or treated for 24 hours with 1 mg/ml FV albumin. FV MGCM did not induce OPC death. C. OPCs were cultured alone (25,000 cells/well) or with addition of microglia (25,000 cells/insert) in 0.4  $\mu$ m pore cell inserts above the OPCs for 24 hours and cell viability was assessed by live-dead staining. Cells were untreated or treated with 1 mg/ml FV albumin. FV albumin induced OPC death in the presence of microglia, although not to the same extent as when FV albumin was directly administrated to OPCs alone (A). D. Microglia and OPCs were co-cultured in contact. The co-cultures were left untreated or were treated with 1 mg/ml FV albumin for 24 hours. FV albumin did not induce OPC death in the presence of microglia. Since FV albumin was shown to be directly toxic to OPCs (A), microglia protected OPCs from FV albumin induced death. Microglia were activated by FV, as confirmed by their morphology. Isolectin BSI-B<sub>4</sub> was used to selectively stain microglia green and distinguish them from OPCs. The fluorescence microscopy fields show FV treated microglia and OPCs in co-culture without or with phase contrast stained with Hoechst 33342 (blue), PI (red) and BSI-B<sub>4</sub> (green). Details of two activated microglia can be seen. The microglial cell on the right is phagocytic (spongiform). A bipolar OPC can be seen in close proximity to the microglia. In all cases the graphs (i) represent the percentage of the total number of cells in OPC cultures that were late apoptotic following Hoechst 33342 (blue) and PI (red) staining as assessed by live-dead staining, while in (ii) representative fluorescence microscopy images of cell viability of control or treated cells are presented. The values in all graphs represent the mean  $\pm$  SEM of three experiments where four fields of each coverslip were counted. In A(i) all treated groups were compared with the control using one way ANOVA with Tukey's post test. Student's t tests were performed between the two groups compared in all other occasions. Levels of significance were ns  $p>0.05$ , \*  $p<0.05$ , \*\*  $p<0.01$ , \*\*\*  $p<0.001$ . Scale bar in all images is 20  $\mu$ m.





**Figure 5.3.1.2 Microglia internalised FITC conjugated albumin**

Primary microglia were incubated with FV albumin for 2 hours. Following incubation 1 mg/ml FITC conjugated albumin (Alb-FITC; green) was added for 0, 30, or 60 minutes at 37°C to assess albumin internalisation by activated microglia. Images of microglial uptake of Alb-FITC were captured at all time points. All nuclei were stained with Hoechst (blue). Green inclusions were obvious in the microglial cytoplasm after 30 or 60 minute incubation with Alb-FITC. Cells were also incubated with Alb-FITC for 60 min at 4°C to prevent internalisation and to ensure that the observed fluorescence was due to internalisation and not binding. Some cells were stained with FITC conjugated isolectin BSI-B<sub>4</sub> to selectively stain microglia, view their morphology and confirm cell culture purity. Scale bar = 20 µm.



### 5.3.2 Effect of fibrinogen on OPCs

Since fibrinogen (FG) enters the brain following BBB disruption in MS (Gay et al., 1997; Gveric et al., 2001), and since it was shown that FG activated microglia *in vitro* (Figures 5.2.3 and 5.2.4), the direct effect of FG on oligodendrocyte lineage cell toxicity was investigated, as well as the effect of FG activated microglia on oligodendrocyte cell toxicity.

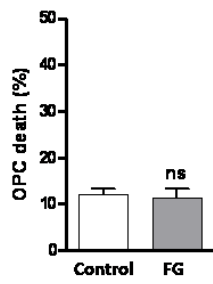
Initially, OPCs were incubated directly with 2.5 mg/ml FG for 24 hours. FG was not toxic to OPCs as assessed by live-dead staining (Figure 5.3.2 A). Furthermore, incubation with microglial-conditioned medium from microglia incubated with 2.5 mg/ml FG for 24 hours (FG-MGCM) was protective to OPCs (Figure 5.3.2 B). This effect was attributed to soluble factors secreted by microglia activated with FG, since untreated MGCM did not protect OPCs in culture.

Conversely, when microglia were cultured above OPCs in 0.4  $\mu$ m pore inserts that only allowed soluble factor transport but no cell contact, 2.5 mg/ml FG addition in the medium in both compartments for 24 hours induced OPC death (Figure 5.3.2 C). Since FG was not directly toxic to OPCs (Figure 5.3.2 A), OPC death was attributed to the activation of microglia by FG. When microglia and OPCs were co-cultured in contact at a ratio of approximately 3:2 and the co-cultures were treated with 2.5 mg/ml FG for 24 hours, OPC death was induced. As when microglia were cultured on inserts above the OPCs, the toxicity was attributed to FG activated microglia (Figure 5.3.2 D). The OPC death induced in the MG-OPC co-cultures was significantly more than the death induced when using inserts to separate microglia from contact with OPCs, indicating that contact between the microglial and OPC populations enhanced the toxic effect of microglia to OPCs (Figure 5.3.2 C,D). The effect of FG on OPCs, which was not directly toxic to OPCs, was protective when FG-MGCM was applied on OPCs and was toxic when the two cell populations were in contact resembled the effect of LPS on OPCs (Figures 4.3.3 and 4.3.4). This response indicated that the action of FG on microglia is mediated by membrane receptor binding.

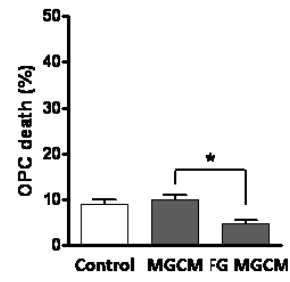
### Figure 5.3.2 Fibrinogen activated microglia were toxic to OPCs

A. OPCs cultured alone were incubated with 2.5 mg/ml FG for 24 hours. FG was not toxic to OPCs. B. OPCs cultured alone were incubated with 50% MGCM for 24 hours. Microglia were left untreated or treated for 24 hours with 2.5 mg/ml FG. FG-activated MGCM was protective to OPCs. C. OPCs were cultured alone (25,000 cells/well) or with addition of microglia (25,000 cells/insert) in 0.4  $\mu$ m pore cell inserts above the OPCs for 24 hours and cell viability was assessed by live-dead staining. Cells were untreated or treated with 2.5 mg/ml fibrinogen. FG induced OPC death in the presence of microglia, indicating that fibrinogen induced microglial activation was toxic for OPCs. D. Microglia and OPCs were co-cultured in contact at a ratio of approximately 3:2. The co-cultures were left untreated or were treated with 2.5 mg/ml FG for 24 h. FG induced OPC death in the presence of microglia, in contrast to when it was directly administered to OPCs (A). Isolectin BSI-B<sub>4</sub> was used to selectively stain microglia green and distinguish them from OPCs. The fluorescence microscopy fields show FG activated microglia and OPCs in co-culture without or with phase contrast stained with Hoechst 33342 (blue), PI (red) and BSI-B<sub>4</sub> (green). Microglia were activated with FG, as confirmed by their morphology. In all cases the graphs (i) represent the percentage of the total number of cells in OPC cultures that were late apoptotic following Hoechst 33342 (blue) and PI (red) staining as assessed by live-dead staining, while in (ii) representative fluorescence microscopy images of cell viability of control or treated cells are presented. The values in all graphs represent the mean  $\pm$  SEM of three experiments where four fields of each coverslip were counted. Student's t tests were performed between the two groups compared in all occasions. Levels of significance were ns  $p > 0.05$ , \*  $p < 0.05$ , \*\*  $p < 0.01$ . Scale bar in all images is 20  $\mu$ m.

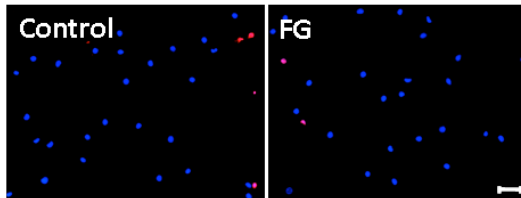
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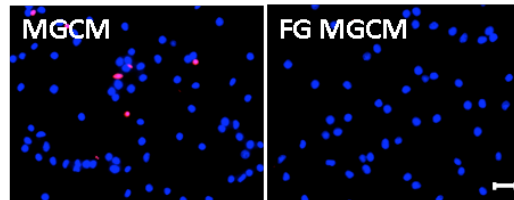
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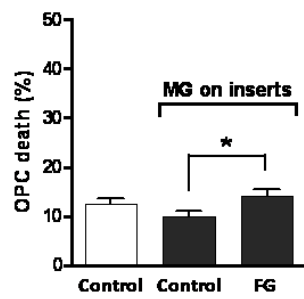
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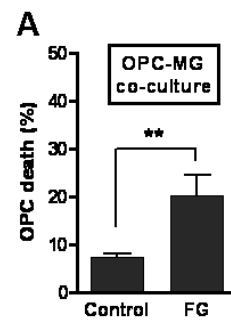
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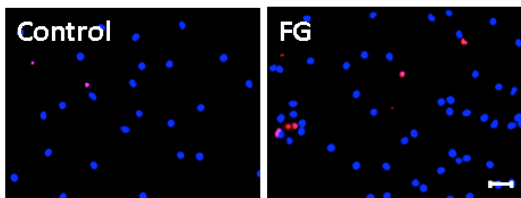
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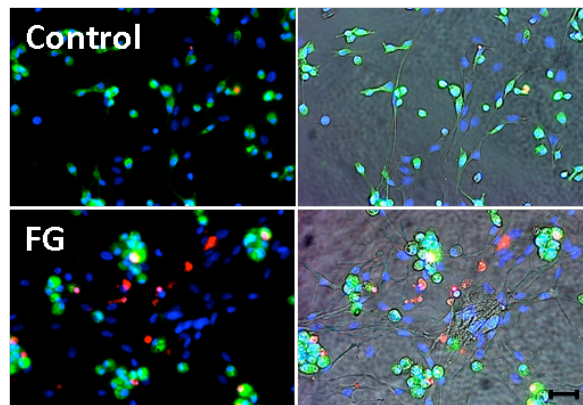
**Di**



**Cii**



**Dii**



### 5.3.3 Effect of fibrin on OPCs

Since fibrin (FN) enters the brain following BBB disruption in MS (Claudio et al., 1995; Wakefield et al., 1994) and since it was shown that FN activated microglia *in vitro* (Figures 5.2.3 and 5.2.4), the direct effect of FN on oligodendrocyte lineage cell toxicity was investigated, as well as the effect of FN activated microglia on oligodendrocyte cell toxicity.

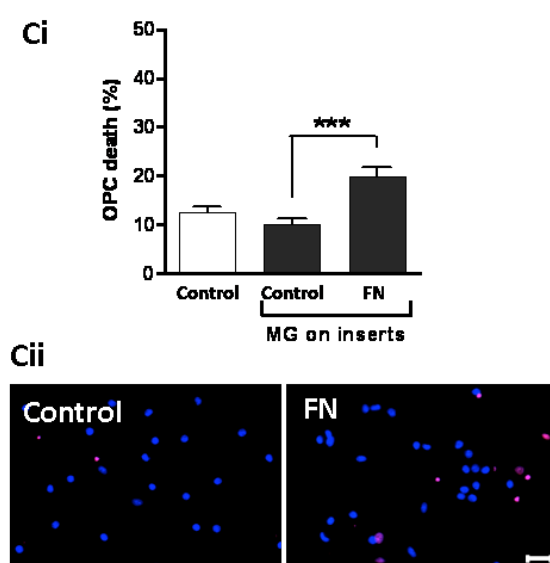
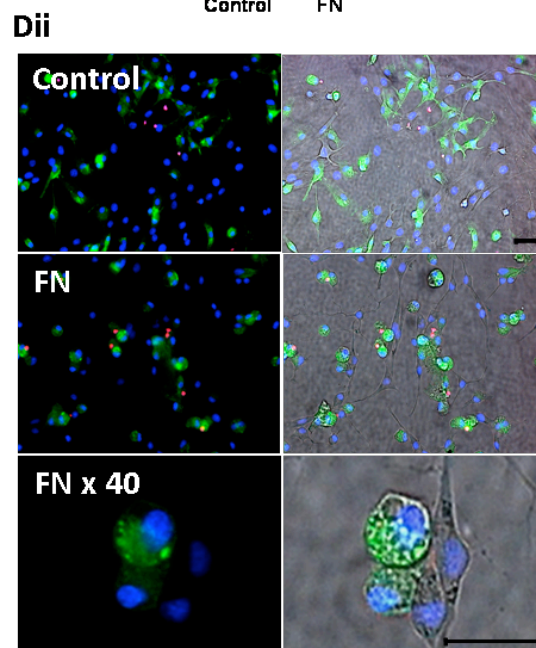
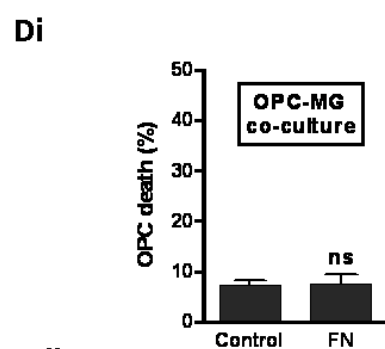
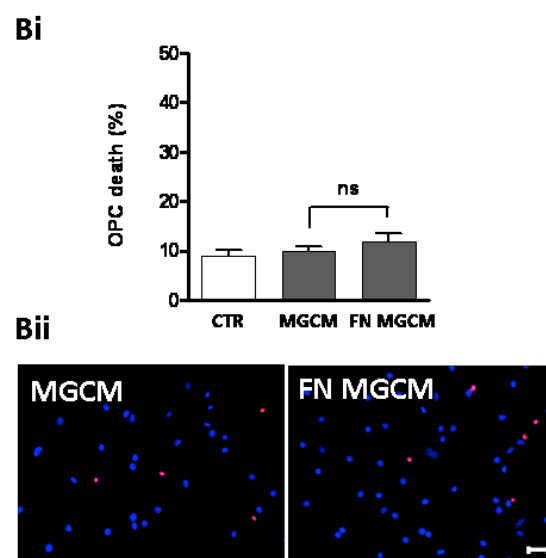
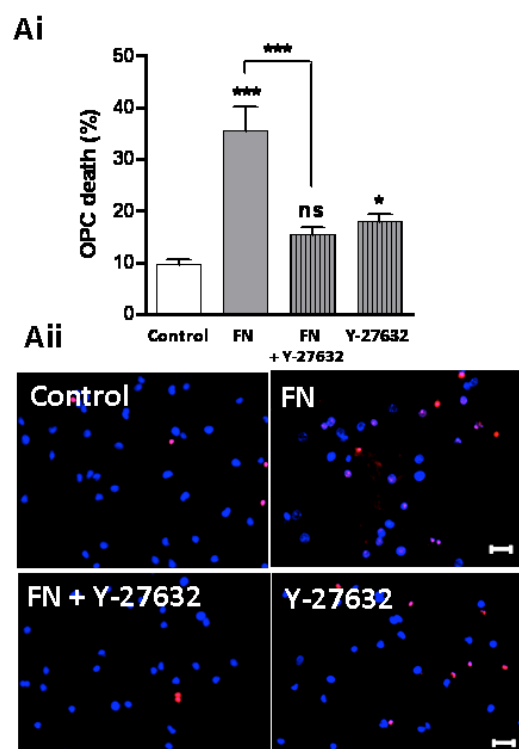
Initially, OPCs were incubated directly with 1 mg/ml FN for 24 hours. FN was significantly toxic to OPCs as assessed by live-dead staining (Figure 5.3.3.1 A). OPCs were incubated with 50  $\mu$ M of the ROCK inhibitor Y-27632, which was able to reverse FN-induced toxicity. OPCs were preincubated for 24 hours with 50  $\mu$ M of the ROCK inhibitor Y-27632 (Maekawa et al., 1999) and then FN was added for another 24 hours. Cells were also treated with Y-27632 for 48 hours to assess Y-27632 toxicity. ROCK inhibition by Y-27632 prevented FN induced OPC death. The ROCK inhibitor Y-27632 was significantly toxic to the cells when applied alone, but interestingly, in the presence of FN, its toxicity was no longer statistically significant (Figure 5.3.3.1 A).

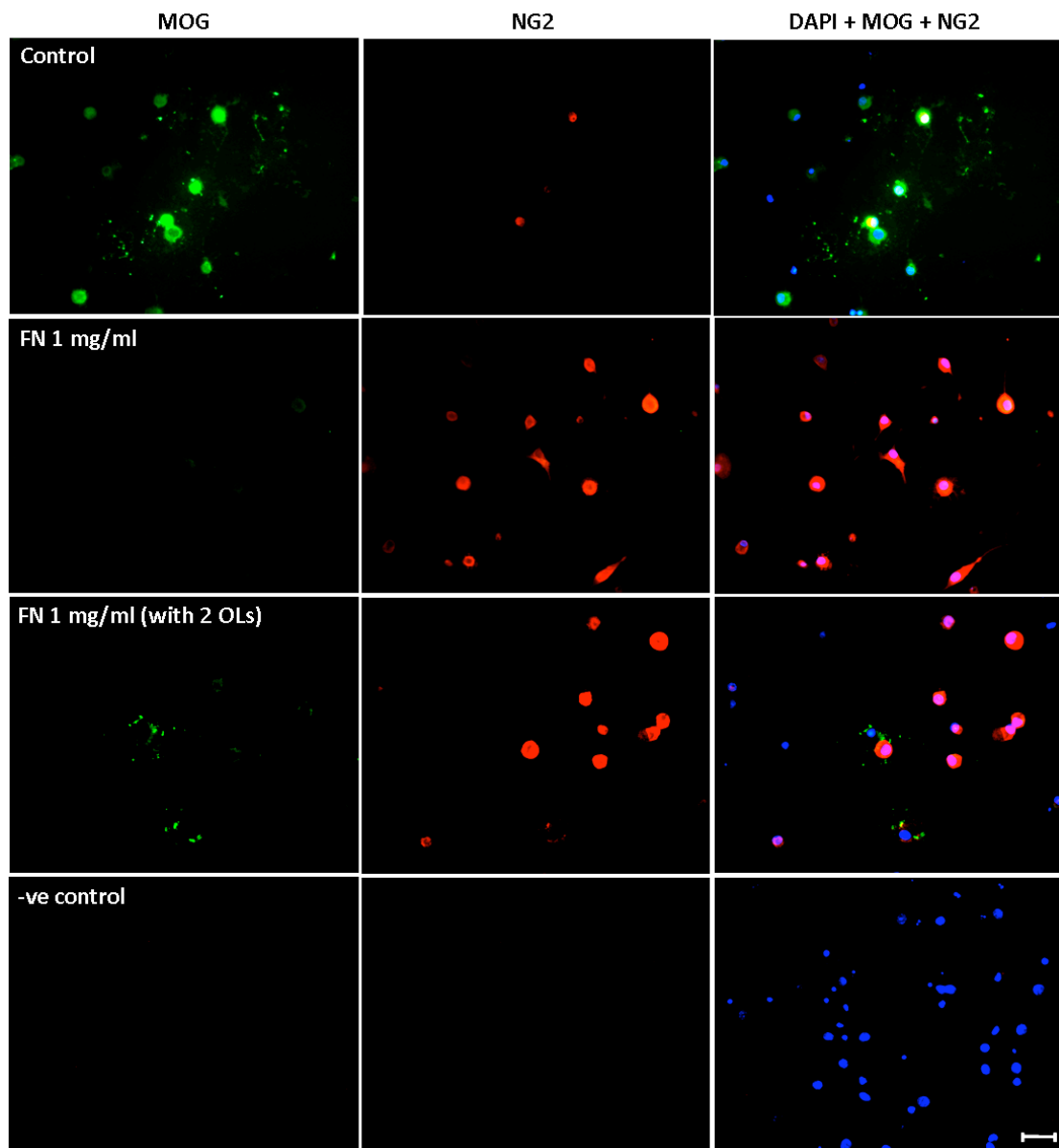
To study the effect of FN activated microglia on OPCs, microglial-conditioned medium (MGCM) from microglia activated with 1 mg/ml FN (potent microglial activator, see Figures 5.2.3 and 5.2.4) was applied to OPCs at a 1:1 ratio with OPC culture medium for 24 hours. FN activated MGCM was not toxic to OPCs after 24 hour treatment (Figure 5.3.3.1 B). When microglia were cultured above OPCs in 0.4  $\mu$ m pore inserts that only allowed soluble factor transfer to OPCs, FN induced OPC death although not to the same extent as when applied directly on the OPCs in the absence of microglia in the culture system (Figure 5.3.3.1 C). Interestingly, when the two cell populations were cultured together and were in contact, administration of FN did not induce OPC death (Figure 5.3.3.1 D). The presence of microglia is able to protect OPCs from FN induced death especially when the cells are in close proximity (co-culture). The spongiform morphology of FN activated microglia in the co-cultured (Figure 5.3.3.1 D) suggests that they internalise FN, thus relieve OPCs from its toxic effects.

When OPCs were left to mature into oligodendrocytes in the presence of FN, it was noticed that they did not differentiate into myelin protein expressing oligodendrocytes within 6 DIV, which is the time required for cells *in vitro* to start expressing myelin antigens (see 3.3.3). The cells rather persisted in a bipolar stage or a late progenitor stage with a few processes. Control and 1 mg/ml FN treated OPCs were fixed after 6 DIV and were immunostained for the myelin specific marker MOG and OPC specific marker NG2. It was shown that only in control cultures OPCs differentiated into MOG expressing oligodendrocytes after 6 days *in vitro* (Figure 5.3.3.2). This was also the case when OPCs were cultured in the presence of microglia, where the persistence in the progenitor state was more obvious, since microglia prevented FN-induced toxicity to OPCs and allowed them to be morphologically distinguished from BSI-B<sub>4</sub> stained microglia in co-culture. FV and FG had no effect on oligodendrocyte maturation (Figure 5.3.3.3 A). This was also confirmed with western blotting of co-cultures for the expression of the mature oligodendrocyte marker MBP in the presence or absence of FN, FG or FV. Indeed only FN was able to prevent OPC maturation and thus MBP expression (Figure 5.3.3.3 B).

### Figure 5.3.3.1 Activated microglia protected OPCs from fibrin-induced toxicity

A. OPCs cultured alone were incubated with 1 mg/ml FN for 24 hours or preincubated for 24 hours with 50  $\mu$ M of the ROCK inhibitor Y-27632 and then FN was added for another 24 hours. Cells were also treated with Y-27632 for 48 hours to assess Y-27632 toxicity. FN induced significant OPC death. ROCK inhibition by Y-27632 prevented FN induced OPC death. The ROCK inhibitor Y-27632 was also significantly toxic to the cells, although when applied together with FN, its toxicity was no longer statistically significant. B. OPCs cultured alone were incubated with 50% MGCM for 24 hours. Microglia were untreated or treated for 24 hours with 1 mg/ml FN. FN activated MGCM was not toxic to OPCs. C. OPCs were cultured alone (25,000 cells/well) or with addition of microglia (25,000 cells/insert) in 0.4  $\mu$ m pore cell inserts above the OPCs for 24 hours and cell viability was assessed by live-dead staining. Cells were untreated or treated with 1 mg/ml FN. FN induced OPC death in the presence of microglia on inserts. D. Microglia and OPCs were co-cultured in contact. The co-cultures were left untreated or were treated with 1 mg/ml FN for 24 hours. FN did not induce OPC death in the presence of microglia, in contrast to when it was directly administered to OPCs (A). The presence of activated microglia protected OPCs against direct FN toxicity. The fluorescence microscopy fields show FN treated microglia and OPCs in co-culture without or with phase contrast stained with Hoechst 33342 (blue), PI (red) and BSI-B<sub>4</sub> (green). Isolectin BSI-B<sub>4</sub> was used to selectively stain microglia green and distinguish them from OPCs. Details of two activated phagocytotic (spongiform) microglia (green – BSI-B<sub>4</sub>) can be seen. Two bipolar OPCs can be seen in close proximity to the microglia. In all cases the graphs (i) represent the percentage of the total number of cells in OPC cultures that were late apoptotic following Hoechst 33342 (blue) and PI (red) staining as assessed by live-dead staining, while in (ii) representative fluorescence microscopy images of cell viability of control or treated cells are presented. The values in all graphs represent the mean  $\pm$  SEM of three experiments where four fields of each coverslip were counted. In A(i) all treated groups were compared with the control using one way ANOVA with Tukey's post test. Student's t tests were performed between the two groups compared in all other occasions. Levels of significance were ns  $p>0.05$ , \*  $p<0.05$ , \*\*\*  $p<0.001$ . Scale bar in all images is 20  $\mu$ m.

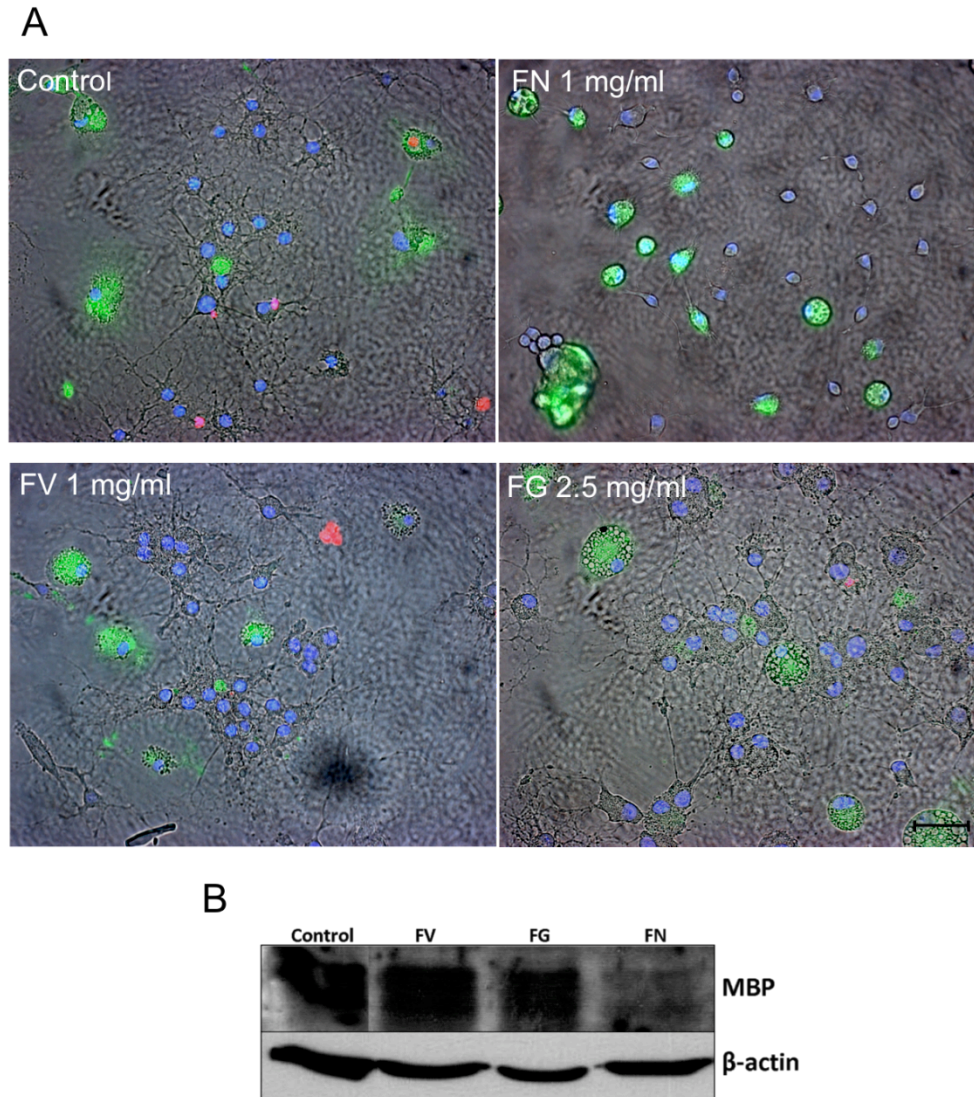




**Figure 5.3.3.2 Fibrin inhibited OPC maturation into oligodendrocytes**

OPCs were cultured for 6 days *in vitro* in the presence of 1 mg/ml FN. After 6 DIV control and FN treated OPCs were fixed and immunostained for the myelin specific marker myelin oligodendrocyte protein (MOG; green), the OPC specific marker NG2 (red) and DAPI to stain all nuclei (blue). Negative control cultures were also stained omitting the primary antibody step. In the presence of FN for 6 DIV, OPCs did not differentiate into myelin protein expressing oligodendrocytes, but rather persisted in a bipolar or late progenitor stage with few processes. Representative fluorescence images of immunostaining where control, FN and negative control cultures were stained can be viewed. Scale bar = 20  $\mu$ m. The values shown represent the mean  $\pm$  SEM of three experiments.





**Figure 5.3.3.3 Fibrin inhibited OPC maturation into oligodendrocytes in the presence of microglia**

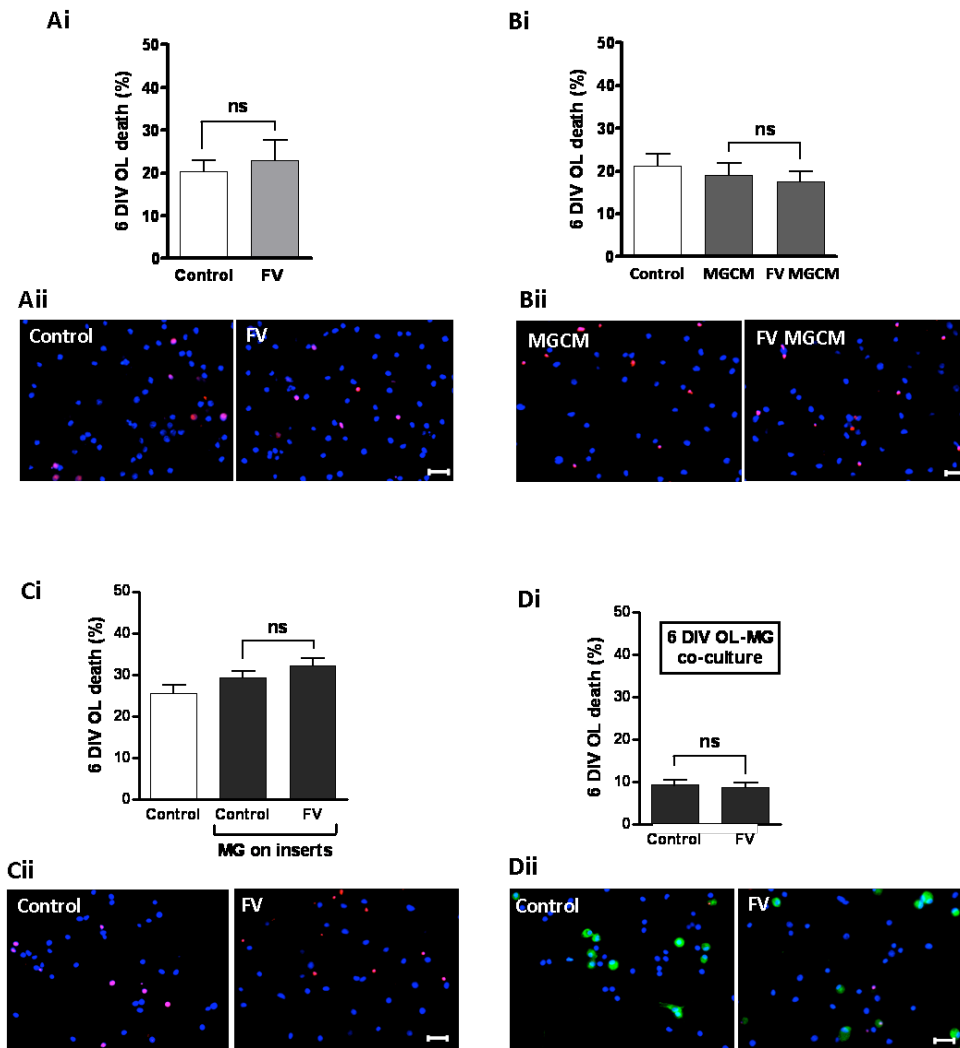
Microglia and OPCs were co-cultured in contact at a ratio of approximately 3:2 for 6 days *in vitro* in the presence of 1 mg/ml FN, 1 mg/ml FV or 2.5 mg/ml FG. After 6 DIV OPCs in control, FV and FG treated cultures differentiated into mature oligodendrocytes. FN treated OPCs did not differentiate into mature oligodendrocytes, but rather persisted in a bipolar or late progenitor stage with a few processes. A. Representative fluorescence microscopy images with phase contrast of MG-OPC co-cultures after 6 DIV. All nuclei were stained with Hoechst (blue), whilst late apoptotic and necrotic nuclei were stained with PI (red). Isolectin BSI-B<sub>4</sub> was used to selectively stain microglia green and distinguish them from OPCs. Scale bar = 20 μm. The values shown represent the mean ± SEM of three experiments. B. Western blot of MBP expression of 6 DIV MG-OPC co-cultures after treatment with FN, FV, or FG. Only FN treated OPCs did not express the mature oligodendrocyte marker MBP after 6 DIV. β-actin was used as a loading control.

## **5.4 Effect of BBPs on 6 DIV mature oligodendrocytes**

### **5.4.1 FV albumin did not affect 6 DIV oligodendrocyte viability**

The effect of FV was studied on 6 DIV oligodendrocytes. The direct effect of FV on 6 DIV oligodendrocyte toxicity was investigated, as well as the effect of FV activated microglia on 6 DIV oligodendrocyte toxicity.

Initially, 6 DIV oligodendrocytes were incubated directly with 1 mg/ml FV for 24 hours. FV was not toxic or protective to OPCs as assessed by live-dead staining (Figure 5.4.1 A). To study the effect of FV activated microglia on 6 DIV oligodendrocytes, microglial-conditioned medium activated with 1 mg/ml FV (FV-MGCM) for 24 hours was applied to oligodendrocytes at a 1:1 ratio with oligodendrocyte culture medium for 24 hours. FV activated MGCM was not toxic or protective to 6 DIV oligodendrocytes after 24 hour treatment (Figure 5.4.1 B). When microglia were cultured above 6 DIV oligodendrocytes in 0.4  $\mu\text{m}$  pore inserts that only allowed soluble factor transport, or when the two cell populations were cultured together and were in contact, administration of FV did not induce 6 DIV oligodendrocyte death (Figure 5.4.1 C,D). Collectively, FV did not have any effect on the viability of mature oligodendrocytes in any of the experimental settings in which it was studied.



**Figure 5.4.1 FV albumin did not affect 6 DIV oligodendrocyte viability**

6 DIV oligodendrocytes were cultured alone (25,000 cells/well; A, B), with addition of microglia (20,000 cells/insert) in 0.4  $\mu$ m pore cell inserts above the oligodendrocytes for 24 hours (C) or were co-cultured in contact with microglia at a ratio of approximately 3:2. Cell viability was assessed by live-dead staining. Cells were left untreated or treated with 1 mg/ml FV. FV did not affect 6 DIV oligodendrocyte viability when administered to the cells directly (A), when used to condition microglial conditioned medium for 24 hours and then applied to the oligodendrocytes (B), when added to 6 DIV oligodendrocytes in the presence of microglia in inserts (C) or in the presence of microglia in contact with the oligodendrocytes (D). In all cases the graphs (i) represent the percentage of the total number of cells in OPC cultures that were late apoptotic following Hoechst 33342 (blue) and PI (red) staining as assessed by live-dead staining, while in (ii) representative fluorescence microscopy images of cell viability of control or treated cells are presented. Isolectin BSI-B<sub>4</sub> was used to selectively stain microglia green and distinguish them from oligodendrocytes (Dii). Scale bar = 20  $\mu$ m. The values shown represent the mean  $\pm$  SEM of three experiments where three fields of each coverslip were counted. Student's t tests were performed to compare groups as indicated by bars. Level of significance was  $p > 0.05$ .

#### 5.4.2 Effect of fibrinogen on 6 DIV oligodendrocytes

The effect of FG was studied on 6 DIV oligodendrocytes. The direct effect of FG on 6 DIV oligodendrocyte toxicity was investigated, as well as the effect of FG-activated microglia on 6 DIV oligodendrocyte toxicity.

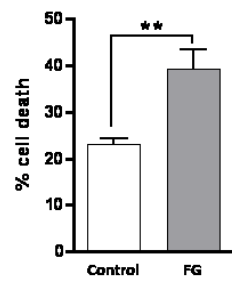
Initially 6 DIV oligodendrocytes were directly incubated with 2.5 or 1 mg/ml FG for 24 hours. FG was toxic to 6 DIV oligodendrocytes at both concentrations as assessed by live-dead staining (Figure 5.4.2 A). 2.5 mg/ml FG were also used to activate microglia, since this concentration was shown to activate microglia *in vitro* and cause iNOS expression (Figures 5.2.3 and 5.2.4). Incubation of 6 DIV oligodendrocytes with microglial-conditioned medium (FG MGCM) was protective to 6 DIV oligodendrocytes (Figure 5.4.2 B). This was also the case when microglia were cultured above 6 DIV oligodendrocytes in 0.4  $\mu$ m pore inserts that only allowed soluble factor transport but no cell contact. 2.5 mg/ml FG addition in the medium for 24 hours was protective to 6 DIV oligodendrocytes (Figure 5.4.2 C). Since FG was protective to 6 DIV oligodendrocytes both in activated MGCM and in the presence of microglia in inserts, soluble molecules are speculated to be involved in this protective effect.

When microglia and 6 DIV oligodendrocytes were cultured in contact, at a ratio of approximately 2:3 in co-culture, treatment with 2.5 mg/ml for 24 hours induced 6 DIV oligodendrocyte death (Figure 5.4.2 D). FG induced death in the MG - 6 DIV oligodendrocyte co-cultures was not as significant as the death induced by direct administration of FG to 6 DIV oligodendrocytes (Figure 5.4.2 A), suggestive of a modulatory role for microglia present in co-culture, although it was inefficient at completely protecting oligodendrocytes. It was also observed that in control cultures microglia were not in contact with oligodendrocytes, while when they were activated they did appear closer to the oligodendrocytes and in between the long processes (Figure 5.4.2 D).

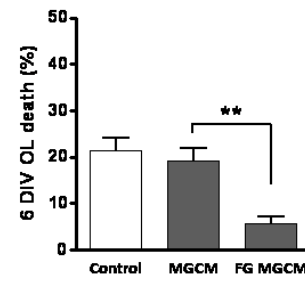
**Figure 5.4.2 Fibrinogen was directly toxic to 6 DIV oligodendrocytes and the presence of microglia was unable to prevent this toxicity**

A. 6 DIV oligodendrocytes cultured alone were incubated with 2.5 mg/ml FG for 24 hours. FG was directly toxic to 6 DIV oligodendrocytes. B. 6 DIV oligodendrocytes cultured alone were incubated with 50% MGCM for 24 hours. Microglia were left untreated or treated for 24 hours with 2.5 mg/ml FG. FG activated MGCM was protective to 6 DIV oligodendrocytes. C. 6 DIV oligodendrocytes were cultured alone (25,000 cells/well) or with addition of microglia (25,000 cells/insert) in 0.4  $\mu$ m pore cell inserts above 6 DIV oligodendrocytes for 24 hours and cell viability was assessed by live-dead staining. Cells were untreated or treated with 2.5 mg/ml FG. FG protected 6 DIV oligodendrocytes in the presence of microglia in inserts. D. Microglia and 6 DIV oligodendrocytes were co-cultured. The co-cultures were left untreated or were treated with 2.5 mg/ml FG for 24 hours. FG induced 6 DIV oligodendrocyte death in the presence of microglia. Isolectin BSI-B<sub>4</sub> was used to selectively stain microglia green and distinguish them from 6 DIV oligodendrocytes. Microglia were activated by FG, as confirmed by their morphology. The representative fluorescence microscopy images of cell viability show 6 DIV oligodendrocytes in co-culture with microglia untreated or treated with FG without or with phase contrast. It was also observed that while in control cultures microglia and mature oligodendrocytes were not in contact, when microglia were activated they did show increased contact with oligodendrocytes. In all cases the graphs (i) represent the percentage of the total number of cells in OPC cultures that were late apoptotic following Hoechst 33342 (blue) and PI (red) staining as assessed by live-dead staining, while in (ii) representative fluorescence microscopy images of cell viability of control or treated cells are presented. Scale bar = 20  $\mu$ m. The values shown represent the mean  $\pm$  SEM of three experiments where four fields of each coverslip were counted. Student's t tests were performed to compare groups as indicated by bars. Levels of significance were \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

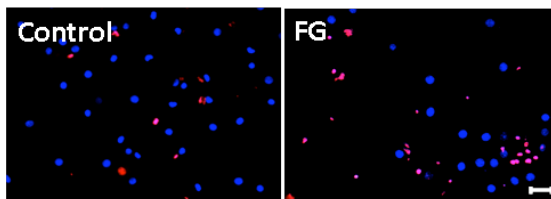
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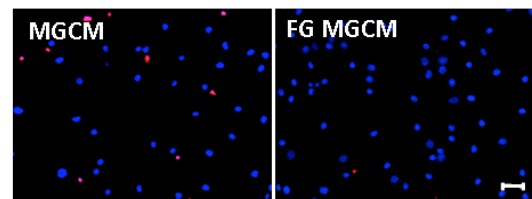
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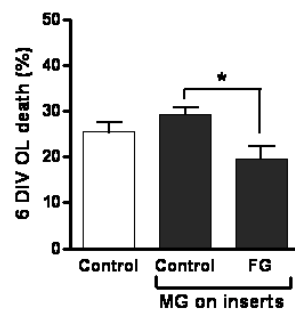
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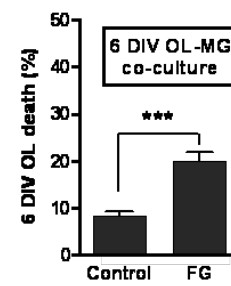
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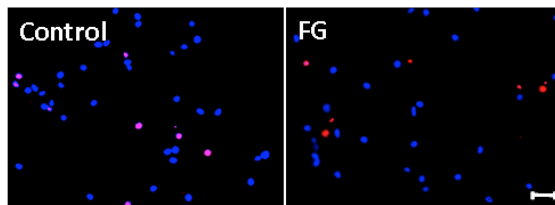
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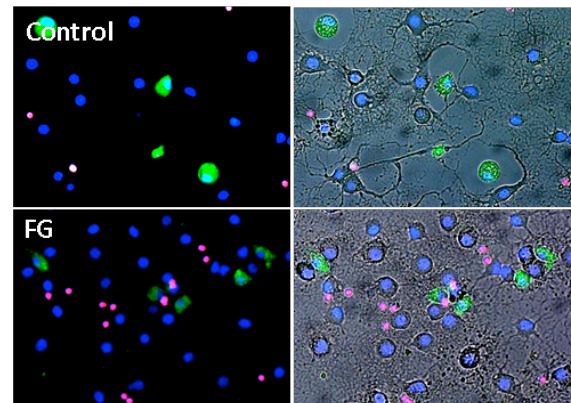
**Di**



**Cii**



**Dii**



### 5.4.3 Effect of fibrin on 6 DIV oligodendrocytes

The effect of FN was studied on 6 DIV oligodendrocytes. The direct effect of FN on 6 DIV oligodendrocyte toxicity was investigated, as well as the effect of FN activated microglia on 6 DIV oligodendrocyte toxicity.

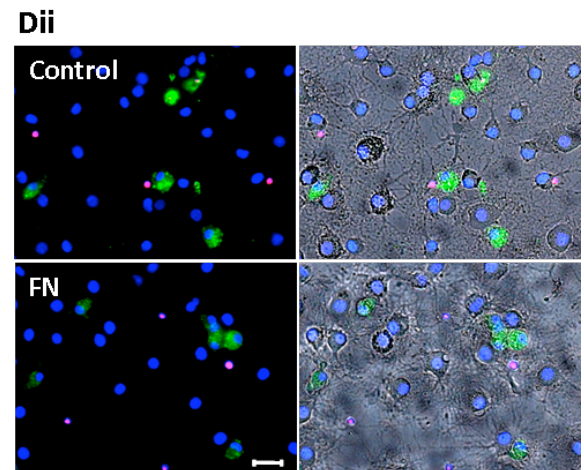
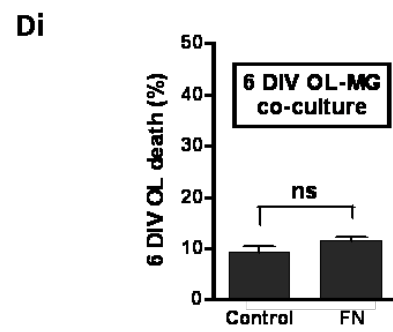
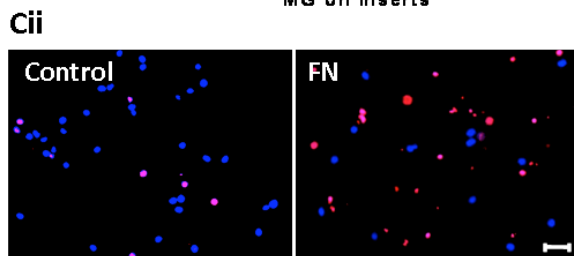
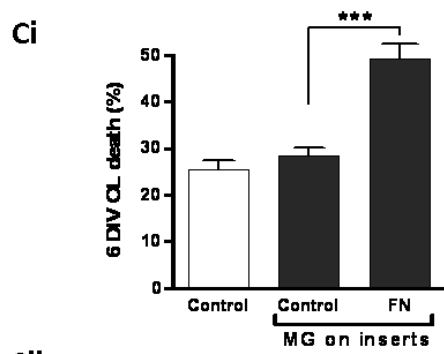
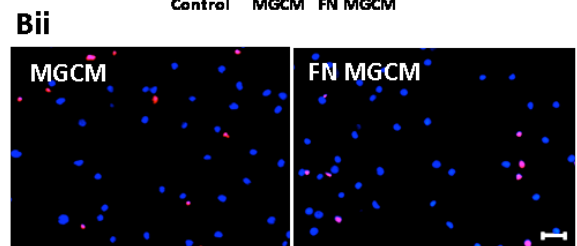
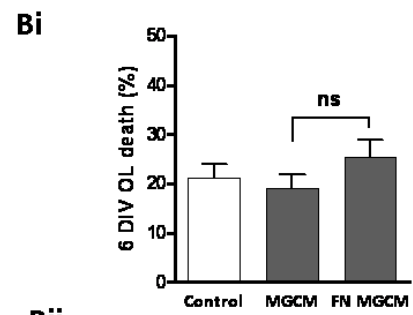
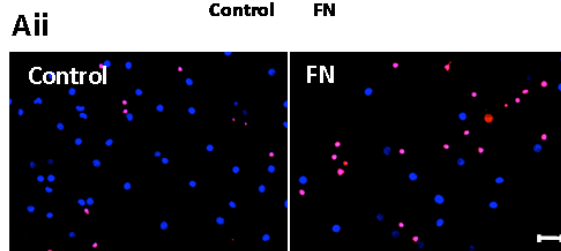
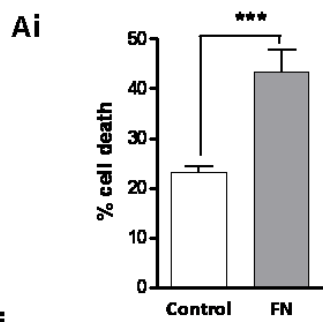
6 DIV oligodendrocytes were directly incubated with 1 mg/ml FN for 24 hours. FN was significantly toxic to 6 DIV oligodendrocytes as assessed by live-dead staining (Figure 5.4.3 A). 1 mg/ml FN was also used to activate microglia, since this concentration was shown to activate microglia *in vitro* and cause iNOS expression (Figures 5.2.3 and 5.2.4). Incubation of 6 DIV oligodendrocytes with microglial conditioned medium (FN MGCM) did not have an effect on 6 DIV oligodendrocyte viability (Figure 5.4.3 B).

In line with the direct administration of FN data (Figure 5.4.3 A), when microglia were cultured above 6 DIV oligodendrocytes in 0.4  $\mu$ m pore inserts that only allowed soluble factor transport but no cell contact, 1 mg/ml FN addition in the medium for 24 hours induced significant 6 DIV oligodendrocyte death (Figure 5.4.3 C). Since FN was also directly toxic to 6 DIV oligodendrocytes (Figure 5.4.3 A), death was attributed to the direct toxicity of FN to 6 DIV oligodendrocytes, which microglia in inserts were unable to reverse. When microglia and 6 DIV oligodendrocytes were co-cultured in contact at a ratio of approximately 2:3 and the co-cultures were treated with 1 mg/ml FN for 24 hours, 6 DIV oligodendrocyte death was prevented (Figure 5.4.3 D). This protective effect of microglia was attributed to their ability to internalise FN and clear the oligodendrocyte environment from toxic FN deposits.

**Figure 5.4.3 Activated microglia protected 6 DIV oligodendrocytes from direct fibrin-induced toxicity**

**A.** 6 DIV oligodendrocytes cultured alone were incubated with 1 mg/ml FN for 24 hours. FN was toxic to 6 DIV oligodendrocytes. **B.** 6 DIV oligodendrocytes cultured alone were incubated with 50% MGCM for 24 hours. Microglia were left untreated or treated for 24 hours with 1 mg/ml FN. FN activated MGCM was toxic to 6 DIV oligodendrocytes. **C.** 6 DIV oligodendrocytes were cultured alone (25,000 cells/well) or with addition of microglia (25,000 cells/insert) in 0.4  $\mu\text{m}$  pore cell inserts above the 6 DIV oligodendrocytes for 24 hours and cell viability was assessed by live-dead staining. Cells were untreated or treated with 1 mg/ml FN. FN induced 6 DIV oligodendrocyte death in the presence of microglia on inserts. **B.** Microglia and 6 DIV oligodendrocytes were co-cultured. The co-cultures were untreated or were treated with 1 mg/ml FN for 24 hours. FN did not induce 6 DIV oligodendrocyte death in the presence of microglia, in contrast to when it was directly administered to 6 DIV oligodendrocytes (**A**). The presence of activated microglia protected 6 DIV oligodendrocytes against direct FN-induced toxicity. Isolectin BSI-B<sub>4</sub> was used to selectively stain microglia green and distinguish them from 6 DIV oligodendrocytes. Microglia were activated by FN, as confirmed by their morphology. The representative fluorescence microscopy images of cell viability show 6 DIV oligodendrocytes in co-culture with microglia untreated or treated with FN without or with phase contrast. In all cases the graphs (i) represent the percentage of the total number of cells in OPC cultures that were late apoptotic following Hoechst 33342 (blue) and PI (red) staining as assessed by live-dead staining, while in (ii) representative fluorescence microscopy images of cell viability of control or treated cells are presented. Scale bar = 20  $\mu\text{m}$ . The values shown represent the mean  $\pm$  SEM of three experiments where four fields of each coverslip were counted. Student's t tests were performed to compare groups as indicated by bars. Levels of significance were ns  $p>0.05$  and \*\*\*  $p<0.001$ .





## 5.5 Discussion

In this chapter the effect of the BBPs FV, FG and FN on microglial activation was studied. It was shown that FV, FG and FN were all potent microglial activators that induced iNOS expression, TNF- $\alpha$  release and morphological changes indicative of phagocytosing microglia. FV, FG and FN were all directly toxic to OPCs or mature 6 DIV oligodendrocytes, whilst the presence of BBP activated microglial conditioned medium or the physical presence of microglia combined with BBP treatment had diverse effects on OPC or 6 DIV oligodendrocyte viability. These findings are summarised in table 5.5.1 and will be discussed in detail. It was also shown that FN had the ability to arrest OPC differentiation into mature oligodendrocytes, a finding with important clinical relevance to MS.

		Direct	MGCM	MG in inserts	MG in contact
LPS	OPCs	<i>no effect</i>	protective	toxic	toxic
	6 DIV OL	<i>no effect</i>	<i>no effect</i>	<i>no effect</i>	toxic
FV albumin	OPCs	toxic	<i>no effect</i>	toxic	<i>no effect</i>
	6 DIV OL	<i>no effect</i>	<i>no effect</i>	<i>no effect</i>	<i>no effect</i>
Fibrinogen	OPCs	<i>no effect</i>	protective	toxic	toxic
	6 DIV OL	toxic	protective	protective	toxic
Fibrin	OPCs	toxic	<i>no effect</i>	toxic	<i>no effect</i>
	6 DIV OL	toxic	<i>no effect</i>	toxic	<i>no effect</i>

**Table 5.5.1 Differential effect of blood borne proteins on oligodendrocytes**

Table of OPC or 6 DIV oligodendrocyte (6 DIV OL) vulnerability to the blood borne proteins (BBPs) FV albumin, fibrinogen and fibrin for 24 hours *in vitro*. The columns represent direct treatment with BBPs (direct), treatment with BBP activated microglial conditioned medium (MGCM), treatment with BBPs in the presence of microglia in cell inserts that did not allow cell contact (MG in inserts) and treatment with BBPs when the two cell populations were co-cultured (MG in contact). The BBPs were either toxic, protective, or had no significant effect on oligodendrocyte viability as assessed by live dead staining.

### **5.5.1 Microglial activation in serum free medium**

In the presence of serum in the culture medium, microglial activation by FV (Figure 5.2.1) and FG (Dr Emma East, unpublished data) did not result in iNOS expression, in contrast to when the cells were cultured in serum free medium. Also, TNF- $\alpha$  release following FG activation in serum free medium was substantially more than TNF- $\alpha$  release in serum containing medium (Piers et al, unpublished data). Such is also the case for FV. FV released equal amounts of TNF- $\alpha$  with LPS in serum free medium (Figure 5.2.5), while when microglia were cultured in serum free medium for only 2 hours of a total 24 hour incubation, TNF- $\alpha$  levels released were significantly lower than LPS induced levels (Hooper et al. 2009a). These results suggest that the presence of serum in the culture medium desensitizes the receptors to which the activator proteins bind, or masks the sites of binding, thus not allowing protein-receptor interactions. It has been shown that serum affects the resting state of microglia, for example serum causes cyclic AMP increase in cultured microglia (Patrizio et al., 1996). Even though the mechanisms for such serum-dependent or independent responses are not well described, serum free microglial cell culture was preferred when studying extracellular signalling to microglial signalling pathways, to avoid serum interference.

### **5.5.2 Amelioration of FV albumin or fibrin induced toxicity to oligodendrocytes by microglial internalisation**

FV was shown to be directly toxic to OPCs (Figure 5.3.1.1). The presence of microglia in contact with OPCs was able to protect OPCs from FV induced toxicity (Figure 5.3.1.4) more potently than microglia in inserts that were only able to partially ameliorate the toxic effects of FV (figure 5.3.1.3). This data suggested that microglia were able to remove FV from the OPC microenvironment, thus promoting OPC survival when in close proximity to the cells, rather than in inserts that provided a physical barrier between cell populations. This was further supported by data showing that FV could be actively taken up by microglia *in vitro* (Figure 5.3.1.5). FV has been shown to increase intracellular calcium and induce microglial proliferation by upstream Src tyrosine kinase and phospholipase C-mediated pathways (Hooper et

al., 2005). Src signalling was recently involved in fibrillary A $\beta$  internalisation by microglia (Reed-Geaghan et al., 2009). It was also shown that mice deficient in all Src kinases exhibited impaired bacterial clearance following infection (Paul et al., 2008).

It is thus proposed that in MS, FV that leaks into the cerebral endothelium following BBB disturbance is toxic to OPCs but not mature oligodendrocytes. This toxicity can be overcome by microglial removal of FV, especially in the inflammatory stage of disease where activated microglia and macrophages clear up the lesions (Nielsen et al., 2009). It is possible though that in chronic lesions persistent activation of microglia may lead to their apoptosis, serving to remove highly reactive and possibly neurotoxic microglia. *In vitro* it was shown that microglial activation resulted in p53 mediated microglial apoptosis (Davenport et al., 2009). The loss of microglia may have adverse consequences for cell survival and recovery, such as in the case of OPCs that are exposed to toxic levels of FV that cannot be removed by microglia.

FN was also toxic to OPCs (Figure 5.3.3.1), similarly but even more potently than FV. FN was toxic to mature oligodendrocytes as well (Figure 5.4.3.1). FN toxicity to OPCs or mature oligodendrocytes could be reversed in the presence of microglia, similarly to FV induced OPC toxicity (Table 5.5.1). This suggests that FN internalisation is initiated following microglial exposure to FN, thus clearing the cell microenvironment and promoting OPC and mature oligodendrocyte survival *in vitro*. It was shown that FN induced activation of microglia *in vitro*, via a fibrin-dependent activation of Akt and Rho signalling, and resulted in cytoskeletal rearrangements and increased phagocytic capacity (Adams et al., 2007). *In vivo*, fibrin depletion in animal models of MS decreased inflammation and remyelination onset, indicating that fibrin exacerbates the inflammatory response in MS (Akassoglou et al., 2004).

### **5.5.3 OPC differentiation arrest and toxicity in the presence of fibrin**

FN enters the brain following BBB disruption in MS (Claudio et al., 1995; Wakefield et al., 1994) ischemia (Schoots et al., 2003) and late stage Alzheimer's (Thomas et al., 1996; Merkle et al., 1996; Melchor et al., 2003). Cells of the nervous system are thus exposed to physiologically irrelevant high concentrations of FN and this may have

adverse effects for disease progression. It was shown here how FN can be directly toxic to both OPCs and mature oligodendrocytes (Figures 5.3.3.1 and 5.4.3.1). It has also been shown that following BBB disturbance FN deposits as an insoluble mesh into the brain and may damage it by preventing neurite outgrowth (Akassoglou et al., 2000). A study in peripheral nerve injury in mice showed that plasminogen activator enhanced nerve regeneration and remyelination after injury by FN deposition removal (Song et al. 2006). Furthermore it was shown that FN regulated Schwann cell differentiation and inhibited remyelination in peripheral nerve injury (Akassoglou et al. 2002).

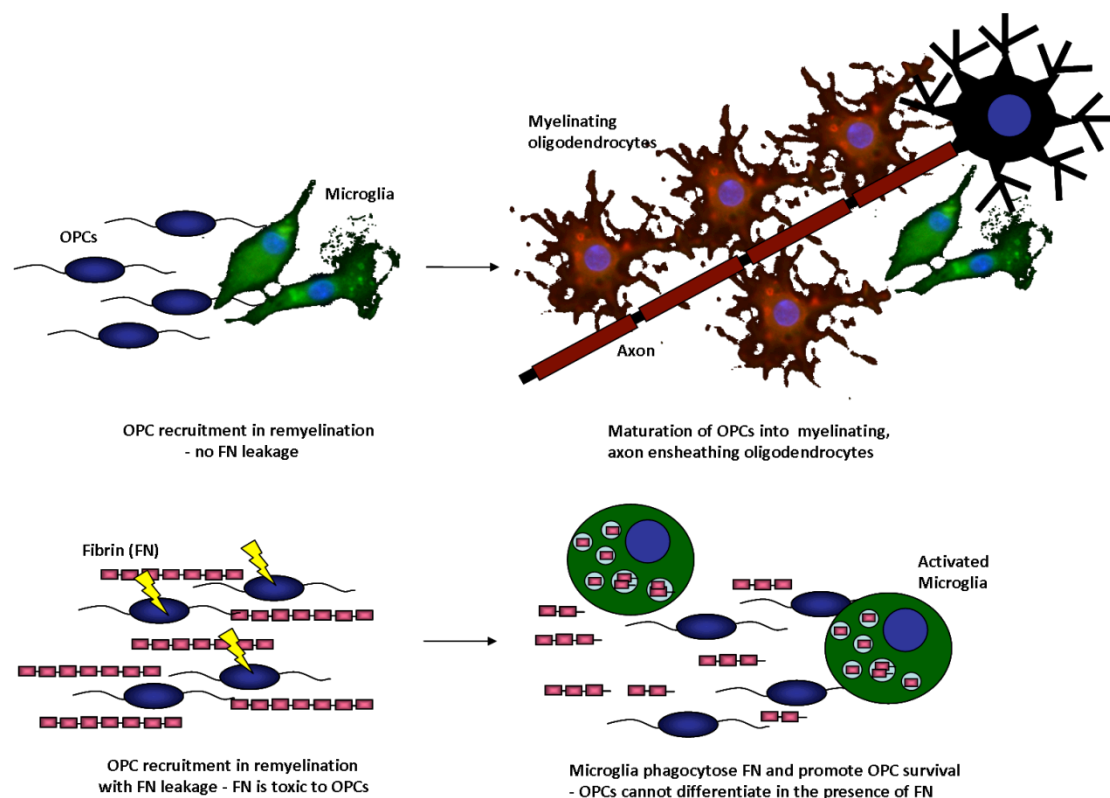
Most of the studies on FN have been performed on spinal cord injury models. Less is known about the effect of FN on CNS cells. Interestingly it was shown here that direct addition of FN in OPC cultures resulted in OPC persistence in a bipolar – precursor form. After 6 days *in vitro*, cells that would have normally differentiated into mature myelinating cells remained in their precursor form (Figure 5.3.3.5). This was also the case when OPCs were cultured in the presence of microglia, that on one hand prevented the toxic effect of FN but on the other hand their presence had no effect on differentiation arrest (Figure 5.3.3.6). This finding has implications for disease pathology in MS and contributes to the explanation of remyelination failure. The presence of FN in MS lesions is inhibitory to remyelination, and thus, strategies that could remove FN from MS lesions or pharmacologically inhibit the intracellular pathways responsible for differentiation arrest could be beneficial for remyelination enhancement and recovery in MS.

In addition to data presented here showing that FN inhibits OPC differentiation, other molecules present in the extracellular space following brain injury or demyelination have also been implicated in OPC differentiation inhibition. The most prominent of these factors is hyaluronan. Hyaluronan is a glycosaminoglycan composed of multiple disaccharide units of glucuronic acid and N-acetylglucosamine. It was found that high molecular weight hyaluronan synthesised by astrocytes accumulated in demyelinated chronic MS lesions and inhibited remyelination by inhibiting OPC maturation (Back et al., 2005). It has also been observed that myelin prevents OPC maturation *in vitro* (Robinson & Miller, 1999) and *in vivo* (Kotter et al., 2006). The demyelinated axons have also been implicated

in inhibiting OPC proliferation by expressing the polysialylated form of the neural cell adhesion molecule NCAM, PSA-NCAM, normally absent from adult brain (Charles et al., 2002). PSA-NCAM was shown to inhibit differentiation of OPCs *in vitro* (Charles et al., 2000). Thus, an explanation for OPC failure to differentiate into mature myelinating oligodendrocytes in chronic MS lesions is that the lesion environment is inhibitory and remyelination failure in MS is more likely due to failure in OPC differentiation than failure in OPC recruitment (Chang et al., 2002). All molecules implicated in OPC differentiation inhibition are high molecular weight polymers, or in the case of myelin lipidic membrane structures. This suggests that there may be a common mechanism by which these high molecular weight molecules can act upon OPCs and preserve them in an immature, proliferating stage. Additionally, FN is the only compound described able to inhibit differentiation that is not derived from cells of the CNS but rather enters the brain following BBB disturbance. Differentiation arrest might serve to preserve an OPC 'pool' for when the environment is more permissive for successful remyelination, while the polymeric structures signal the presence of a non-permissive, or even developing (like in the case PSA-NCAM) environment. The clinical significance of these findings might lead to therapeutic practices aiming at clearing the demyelinating lesions, thus allowing successful differentiation of OPCs into myelinating cells. It was shown *in vivo* that tissue plasminogen activator (tPA), a protease that converts plasminogen to plasmin, which in turn degrades fibrin, protected axons from degeneration and demyelination, which correlated with increased deposition of fibrinogen (Akassoglou et al., 2000; Zou et al., 2006). It should also be noted that Schwann cells expressed tPA in response to injury (Akassoglou et al., 2000), possibly in an attempt to make the environment more permissive to remyelination.

In regards to FN induced toxicity to OPCs, this was reversed by inhibiting ROCK with its specific inhibitor Y-27632 (Figure 5.3.3.1). This was not the case for FV induced toxicity (Figure 5.3.1.1), but this may be due to the fact that FN increased death levels by 30%, whilst FV induced 15% increase in cell death after 24 hours of incubation. The ROCK inhibitor Y-27632 was significantly toxic to the cells (10% above basal levels), so even though when applied together with FN it was able to reduce toxicity (Figure 5.3.3.1), when added to FV treated cells its toxicity may have

interfered with the results. Prevention of FN toxicity by ROCK inhibition in OPCs implicated the Rho-ROCK mediated intracellular pathway in the toxic cascades induced in OPCs. Since ROCK inhibition has already been proposed as a therapeutic strategy in MS due to its anti-inflammatory properties (Honing et al., 2004; Paintlia et al., 2005; Miron et al., 2007) its role in promoting OPC survival following exposure to FN points toward additional beneficial effects in cells other than macrophages.



**Figure 5.5.3.1 The effect of fibrin on OPCs**

Schematic representation of the proposed events occurring in demyelinated sites in MS during successful myelination or in the presence of fibrin (FN). In the top image OPCs differentiate into mature oligodendrocytes that can myelinate neuronal axons. Microglia are present in a ramified 'surveying' state. In the lower image the presence of fibrin is toxic to OPCs. Microglia are activated in response to FN and internalise it, thus promoting OPC survival. In the presence of remaining FN though OPCs are unable to differentiate into mature oligodendrocytes.



#### 5.5.4 Action of FG on oligodendrocyte lineage cells

Similarly to FV and FN, FG enters the brain following BBB disruption and has been detected in MS lesions (Gay et al., 1997; Gveric et al., 2001). FG potently activated microglia, caused iNOS expression (Figures 5.2.3 and 5.2.4) and TNF- $\alpha$  release, although notably at lower levels than TNF- $\alpha$  released following microglial activation with LPS or other BBPs (Figures 5.2.5 and 5.2.6). Adams et al, 2007 also showed that FG could activate microglia by morphological rearrangement of actin and  $\beta$ -tubulin, increased internalisation, RhoA activation and Akt phosphorylation.

Microglial activation and oligodendrocyte toxicity *in vitro* in the presence of FG revealed in this study resembled microglial activation by LPS (Chapter 4), as summarised and compared with the effects of the other BBPs studied in Table 5.5.1. In the case of OPCs, FG was not directly toxic. More interestingly microglial conditioned medium was protective to OPCs, an effect attributed to soluble factors released into the conditioned medium. The physical presence of microglia in inserts or in direct contact with OPCs had the opposite effect to conditioned medium and was toxic to the OPCs. This points towards additional microglial mechanisms being involved in FG mediated toxicity. Although TNF- $\alpha$  blockade was able to ameliorate the toxic effect of LPS activated microglia to oligodendrocytes (figure 4.3.4), this was not tested for FG induced OPC death and thus remains as an open question. The fact that TNF- $\alpha$  levels released from FG activated microglia were significantly lower than TNF- $\alpha$  released from LPS-activated microglia (Figures 5.2.5 and 5.2.6), suggests that if OPC death is TNF- $\alpha$  mediated it is not the amount of TNF- $\alpha$  released that determines cell death but the proximity of the TNF- $\alpha$  releasing microglia to OPCs. Additional short-lived molecules may also be involved in this microglial mediated OPC death.

When microglia were activated with FG the protective effects of MGCM on OPCs or the toxic effects of activated microglia in co-culture with OPCs were similar to those observed following microglial activation with LPS, suggesting that FG may act through a distinct receptor on microglia. It was shown that FG binds to the Mac-1 integrin receptor present on microglia (Adams et al., 2007). The Mac-1 integrin receptor is well described as the integrin ligand for leukocytes (reviewed by Ugarova

& Yakubenko, 2001) and multiple Mac-1 recognition sites have been described for FG (Lishko et al., 2004). In this study binding of FG to Mac-1 receptor on microglia was confirmed, although restricted to just FG and not FN as previously shown (Adams et al., 2007). It was also shown that FG does not signal through the LPS receptor TLR4. This will be discussed in detail in Chapter 6.

FG was directly toxic to 6 DIV oligodendrocytes, in contrast to LPS (see Table 5.5.1). FG activated microglial conditioned medium retained its protective properties towards 6 DIV oligodendrocytes as well. Furthermore when 6 DIV oligodendrocytes were cultured below microglia in inserts, microglia were protective. Conversely, when the two cell populations were cultured in contact, FG was toxic to 6 DIV oligodendrocytes. From this data it can be assumed that FG is directly toxic to 6 DIV oligodendrocytes, whilst microglia secrete factors that can promote oligodendrocyte survival. When the two cell populations are in contact though, microglia in close proximity to 6 DIV oligodendrocytes lose their protective properties. It would thus be therapeutically beneficial if the protective mechanisms activated in microglia were preserved with parallel blockade of toxic pathways.

### **5.5.5 Conclusion**

It was shown here that BBP entry into the CNS following BBB disturbance in MS can act as an additional burden to disease progression. Microglia can internalise foreign compounds in MS lesions, but there are limitations to their phagocytosis capacity and following sustained activation microglia apoptose (Kingham et al., 1999; Nicholas et al., 2002; Hooper & Pocock, 2007; Davenport et al., 2009). It is also becoming increasingly evident that the demyelinated environment in MS lesions is inhibitory to remyelination, so methods promoting lesion clearance could promote remyelination. Breakdown of polymers such as FN accumulating in lesions could potentiate OPC differentiation into newly myelinating oligodendrocytes. Modulation of microglial activation following BBP activation could also be beneficial by promoting release of protective soluble factors and blockade of toxic pathways. Pathways activated following microglial activation with LPS or BBPs will be further studied in Chapter 6 and the receptors to which BBPs bind will be discussed.

## **6. MODULATION OF DIFFERENTIAL MICROGLIAL ACTIVATION**

## 6.1 Introduction

iNOS expression has been extensively used as a marker of *in vitro* microglial activation in the literature (Colasanti et al., 1995; Hooper et al., 2005; Hooper and Pocock, 2007; Pinteaux-Jones et al., 2008), as well as in this study (see 4.2 and 5.2), where BV-2 cells and primary microglia expressed iNOS in response to LPS or the BBPs FV, FG and FN. Since microglia were potently activated by BBPs with differential effects on oligodendrocyte lineage cell viability (Chapter 5), here it was investigated how microglial activation as assessed by consequent iNOS expression could be modulated by blockade of the microglial Rho-ROCK pathway or by selective use of specific mGluR agonists and antagonists. The manipulation of the Rho-ROCK pathway or mGluR pathway modulation were selected based on previous studies on the modulatory effect of those pathways in neuroprotection and neurodegenerative disease (see 5.1.3 and 1.5.3 respectively). Furthermore, based on concerns that microglial activators other than the prototypical pathogen-associated molecular pattern (PAMP) might be contaminated with LPS components (Weinstein et al., 2008), it was attempted to clarify this issue. Additionally, potential receptors for BBPs were studied and the involvement of the LPS receptor TLR4 in BBP induced microglial activation was investigated.

### 6.1.1 The Rho-ROCK intracellular pathway in microglia

As discussed in 5.1.3, ROCK is a Rho-GTP binding kinase with numerous downstream effectors and is actively implicated in regulating actin-myosin contractility. ROCK inhibition has been proposed as a feasible neuroprotective strategy in neurodegeneration (reviewed by Mueller et al., 2005).

The Rho-ROCK pathway exists in all cell types (Nakagawa et al., 1996). In macrophages the Rho-ROCK pathway is involved in cytoskeleton rearrangement and migration (Allen et al., 1997), as well as in complement mediated phagocytosis (Caron & Hall, 1998). In microglia, it was shown that the *Clostridium botulinum* bacterial C3 protein (C3bot) was able to transform the cells towards an activated phenotype and trigger the release of nitric oxide via a Rho and NF- $\kappa$ B, but not a

ROCK dependent pathway (Hoffmann et al., 2008). On the other hand, RhoA was shown to negatively regulate cytokine mediated iNOS expression via the inactivation of NF- $\kappa$ B in transformed brain cell lines (Rattan et al., 2003). The involvement of ROCK mediated pathways in BV-2 cell and microglial iNOS expression following LPS or BBP activation was studied here.

### **6.1.2 Effects of microglial mGluR manipulation**

The therapeutic potential of microglial mGluR manipulation in regards to MS was addressed in 1.5.2. Here the ability of mGluRs to modulate microglial activation was investigated. It has been shown that direct stimulation of the group II mgluR2 receptors in microglia can trigger the release of TNF- $\alpha$ , which in concert with soluble Fas ligand can be neurotoxic (Taylor et al., 2005), it is thus clear that glutamate can act upon microglia and elicit a signalling response. At the same time a neuroprotective role has been attributed to group III mGluRs, again providing evidence that activation of these receptors does alter microglial responses. Manipulation of this regulatory, slow metabotropic response could lead to therapeutic strategies when addressing diseases with a neurodegenerative component.

A recent study showed that treatment with group I receptor mGluR5 agonists inhibited LPS induced microglial activation and NO production via phospholipase C, protein kinase C activation and calcium release (Byrnes et al., 2009b). In this study the effect of a range of mGluR agonists and antagonists on preventing LPS activated microglial induced oligodendrocyte death was studied but no protective effect was shown (Figure 4.3.10). Here, the effect of mGluR agonists and antagonists on iNOS expression following microglial activation with LPS or BBPs will be studied in an attempt to investigate mGluR involvement in microglial signalling pathways.

### **6.1.3 Endotoxin contamination of microglial activators**

It was recently reported that many commercially available reagents, such as plasma-derived proteins, phospholipids and peptides, were contaminated with LPS (Weinstein et al., 2008). Thus, responses attributed to microglial activators other

than bacterial derived substances could actually result from residual LPS in the reagents used. It was shown that the LPS inhibitor polymyxin B (PMX), which binds directly to LPS and antagonises its action (Inada, 1980; Dubor et al., 1986) could partially blunt the effect of the FV reagent used in the present study (Sigma-Aldrich, A6272) as well as the effect of other serum derived proteins on microglial proliferation and metabolic activity. Incubation with PMX reduced FV activated microglial TNF- $\alpha$  release by 73%, whilst NO production was decreased by up to 82%, even though the responses were variable so no statistical significance was observed (Weinstein et al., 2008). In the same study the Pyrochrome endotoxin quantification kit (LAL Pyrochrome, Associates of Cape Cod Incorporated) reported significantly high levels of endotoxin in the FV reagent used in this study ( $>0.43$  EU/ml in 1 mg/ml). Here, it was investigated whether the BBPs used to activate microglia in this study were endotoxin free and whether it is safe to attribute the experimental results obtained to BBPs and not LPS contamination.

## 6.2 ROCK inhibition blocked iNOS expression in microglia

BV-2 cells or primary microglia were incubated for 24 hours with the ROCK inhibitor Y-27632 followed by 24 hour activation with LPS, FV, FG or FN. Lysates were collected and western blotting was performed for iNOS expression visualisation.

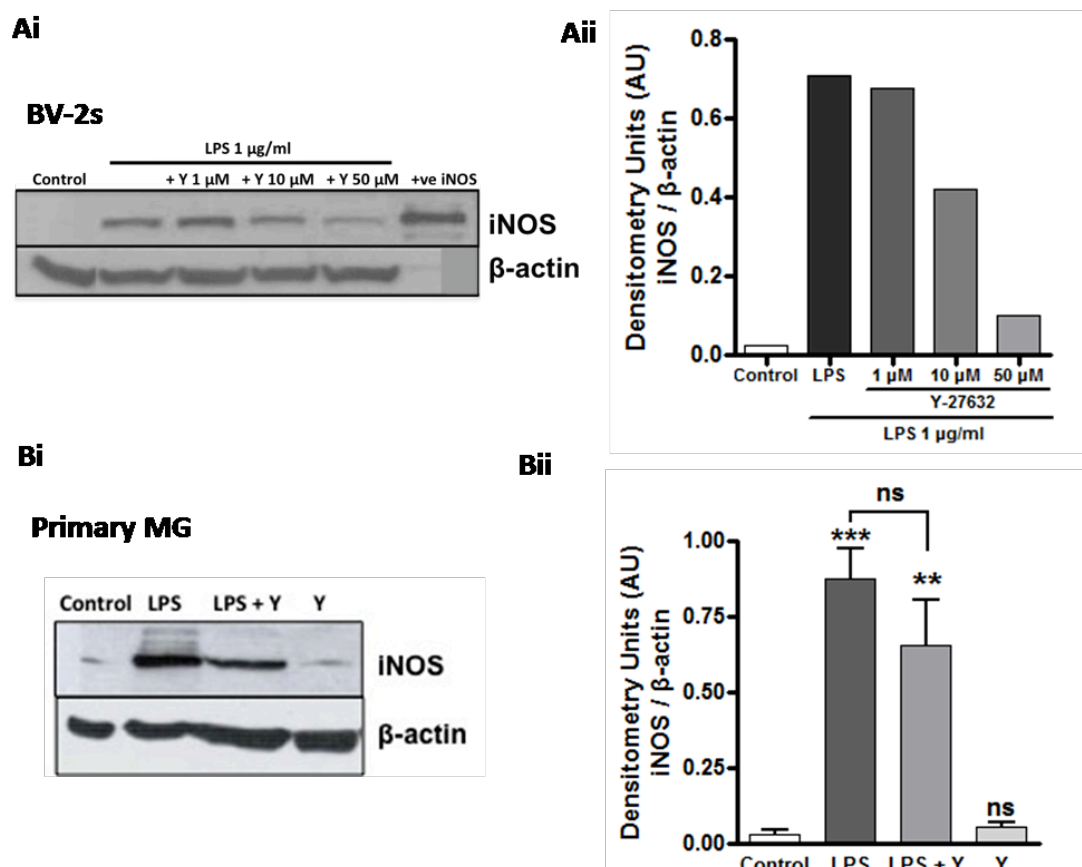
A preliminary experiment in BV-2 cells showed that 1 µg/ml LPS-induced iNOS expression was attenuated by 50 µM Y-27632 (Figure 6.2.1 A). This experiment was repeated in primary microglia, where 50 µM Y-27632 reduced 0.1 µg/ml LPS-induced iNOS in primary microglia, although this reduction did not reach statistical significance (Figure 6.2.1 B). 100 µM Y-27632 was also tested (data not shown), but it did not block iNOS expression. Additionally there were concerns that at 100 µM Y-27632 was toxic to the cells.

It was initially observed that in BV-2 cells 1 mg/ml FV induced iNOS expression that could be attenuated in a dose dependent manner when Y-27632 was applied to the cells (Figure 6.2.2 A). In primary microglia 50 µM Y-27632 was also able to reduce 1 mg/ml FV-induced iNOS expression to more than half, although this reduction was not statistically significant (Figure 6.2.2 B).

In the case of FG, it was initially observed that 50 µM Y-27632 could attenuate 2.5 mg/ml FG-induced iNOS expression in BV-2 cells (Figure 6.2.3 A). In primary microglia there was only partial reduction in iNOS expression, yet 50 µM Y-27632 significantly attenuated iNOS expression (Figure 6.2.3 B). The response of FG activated microglia to Y-27632 incubation resembled the LPS activated microglial response.

Finally, an initial observation in BV-2s showed that 1 mg/ml FN induced iNOS was blocked by 100 µM Y-27632 (Figure 6.2.4 A). BV-2s are more resilient to Y-27632 than primary microglia so 100 µM Y-27632 did not induce cell death. It should be noted though that the Y-27632 batch used was more than 2 months old, and old Y-27632 batches lose their activity even when kept at -80°C. In the case of primary microglia, 50 µM Y-27632 potentially reduced 1 mg/ml FN induced iNOS expression to more than half in primary microglia (Figure 6.2.4 B). The FN activated microglial response to Y-27632 was similar to the microglial response following FV activation. The resemblance between LPS – FG and FV – FN responses points towards different

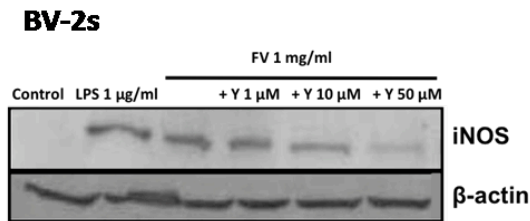
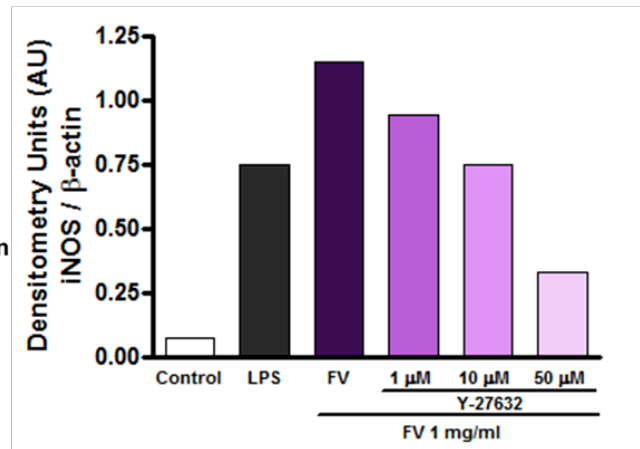
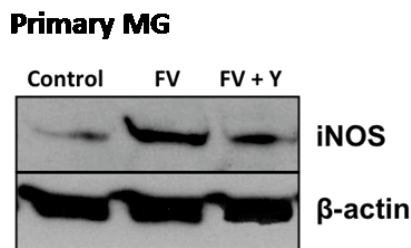
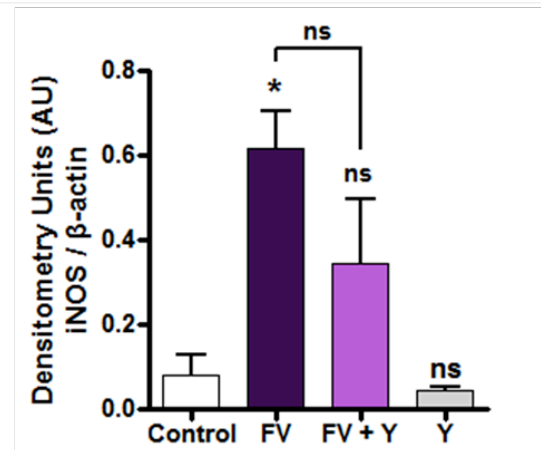
activation pathways that can or cannot be completely attenuated by ROCK inhibition. Differential responses when activated with LPS and FG or FV and FN were observed when microglial toxicity to oligodendrocytes (Chapter 5), was studied.



**Figure 6.2.1 ROCK inhibition attenuated LPS induced iNOS expression in microglia**

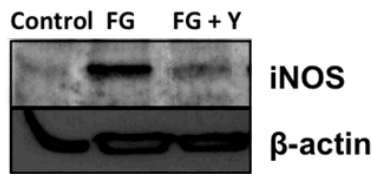
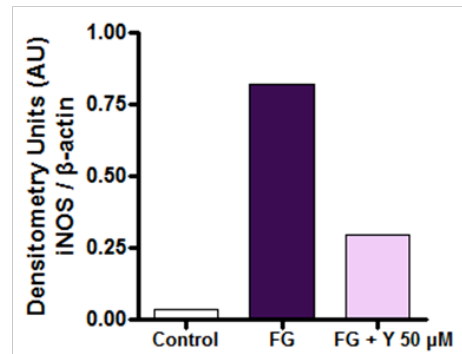
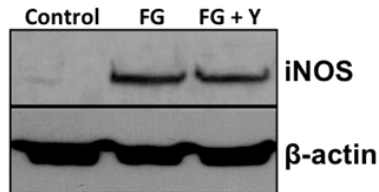
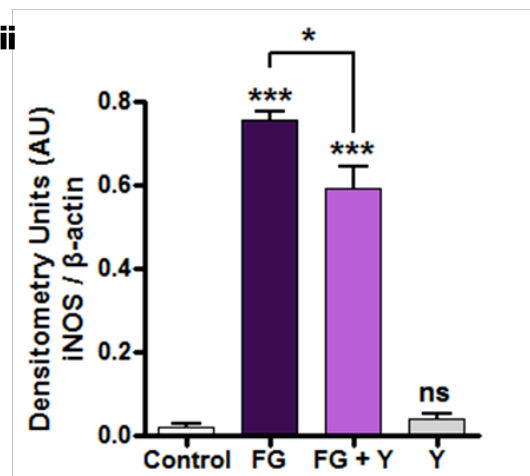
A. Western blot (i) and densitometric analysis (ii) of iNOS expression of BV-2s after incubation for 24 hours with the ROCK inhibitor Y-27632 at (1-50)  $\mu\text{M}$  followed by 24 hour activation with 1  $\mu\text{g/ml}$  LPS in serum free medium. LPS induced iNOS expression in BV-2s showed decreased expression following 50  $\mu\text{M}$  Y-27632 incubation. Commercially available mouse macrophage LPS/IFN $\gamma$  cell lysate (BD Transduction 611473) was used as positive control.  $\beta$ -actin was used as a loading control. B. Representative western blot (i) and densitometric analysis (ii) of iNOS expression of primary microglia after incubation for 24 hours with the ROCK inhibitor Y-27632 at 50  $\mu\text{M}$  followed by 24 hour activation with 0.1  $\mu\text{g/ml}$  LPS in serum free medium. Y-27632 partially attenuated (although not statistically significantly) iNOS expression following LPS activation of microglia.  $\beta$ -actin was used as a loading control. The experiments with primary microglia were repeated in triplicate and one way ANOVA with Tukey's post test was performed. Levels of significance were ns  $p > 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .



**Ai****Aii****Bi****Bii**

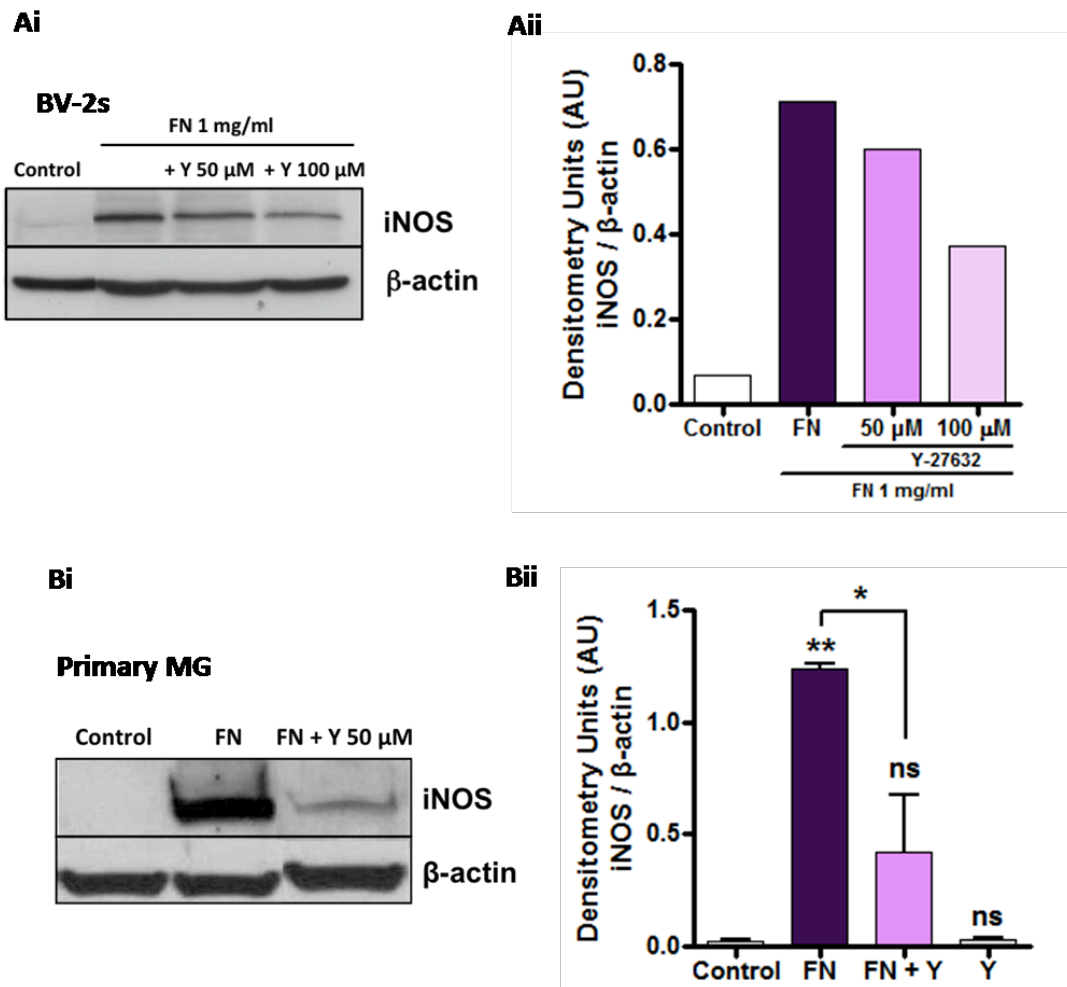
**Figure 6.2.2 ROCK inhibition attenuated FV albumin induced iNOS expression in microglia**

A. Western blot (i) and densitometric analysis (ii) of iNOS expression of BV-2s after incubation for 24 hours with the ROCK inhibitor Y-27632 at (1-50)  $\mu$ M followed by 24 hour activation with 1 mg/ml FV in serum free medium. 50  $\mu$ M Y-27632 was able to attenuate FV induced iNOS expression in BV-2s.  $\beta$ -actin was used as a loading control. B. Representative western blot (i) and densitometric analysis (ii) of iNOS expression of primary microglia after incubation for 24 hours with the ROCK inhibitor Y-27632 at 50  $\mu$ M followed by 24 hour activation with 1 mg/ml FV in serum free medium. Y-27632 appeared able to attenuate (although not statistically significantly) iNOS expression following LPS activation of microglia.  $\beta$ -actin was used as a loading control. The experiments with primary microglia were repeated in triplicate and one way ANOVA with Tukey's post test was performed. Levels of significance were ns  $p > 0.05$ , \*  $p < 0.05$ .

**Ai****BV-2s****Aii****Bi****Primary MG****Bii**

**Figure 6.2.3 ROCK inhibition attenuated fibrinogen induced iNOS expression in microglia**

A. Western blot (i) and densitometric analysis (ii) of iNOS expression of BV-2s after incubation for 24 hours with the ROCK inhibitor Y-27632 at 50  $\mu$ M concentration, followed by 24 hour activation with 2.5 mg/ml FG in serum free medium. 50  $\mu$ M Y-27632 appeared able to attenuate FG induced iNOS expression in BV-2s.  $\beta$ -actin was used as a loading control. B. Representative western blot (i) and densitometric analysis (ii) of iNOS expression of primary microglia after incubation for 24 hours with the ROCK inhibitor Y-27632 at 50  $\mu$ M concentration followed by 24 hour activation with 2.5 mg/ml FG in serum free medium. Y-27632 partially blocked iNOS expression following FG activation of microglia.  $\beta$ -actin was used as a loading control. The experiments with primary microglia were repeated in triplicate and one way ANOVA with Tukey's post test was performed. Levels of significance were ns  $p > 0.05$ , \*  $p < 0.05$ , \*\*\*  $p < 0.001$ .



**Figure 6.2.4 ROCK attenuated fibrin induced iNOS expression in microglia**

A. Western blot (i) and densitometric analysis (ii) of iNOS expression of BV-2s after incubation for 24 hours with the ROCK inhibitor Y-27632 at (50-100)  $\mu$ M concentration, followed by 24 hour activation with 1 mg/ml FN in serum free medium. 100  $\mu$ M Y-27632 appeared able to attenuate FN induced iNOS expression in BV-2s.  $\beta$ -actin was used as a loading control. B. Representative western blot (i) and densitometric analysis (ii) of iNOS expression of primary microglia after incubation for 24 hours with the ROCK inhibitor Y-27632 at 50  $\mu$ M concentration followed by 24 hour activation with 1 mg/ml FN in serum free medium. Y-27632 attenuated iNOS expression following FG activation of microglia.  $\beta$ -actin was used as a loading control. The experiments with primary microglia were repeated in triplicate and one way ANOVA with Tukey's post test was performed. Levels of significance were ns  $p>0.05$ , \*  $p<0.05$ , \*\*  $p<0.01$ .

### 6.3 Modulation of differential microglial activation by mGluRs

To study the effect of mGluR agonists and antagonists on iNOS expression following microglial activation with LPS or BBPs, BV-2 cells or primary microglia were activated with LPS or BBPs for 24 hours in the presence of mGluR agonists and antagonists. The agonists and antagonists were always added 30 minutes before the microglial activators, to allow them to bind to their receptors and initiate cellular responses (Taylor et al., 2002). Lysates were collected following 24 hours of incubation and western blotting was performed on the cytoplasmic fraction.

Before studying the effect of mGluR agonists and antagonists on activated microglia, iNOS expression following 24 hour direct incubation of BV-2 cells with the mGluR agonists or antagonists alone was studied. The mGluR agonists and antagonists used were the group I agonist DHPG (100  $\mu$ M), the group I antagonists AIDA (250  $\mu$ M) and SIB-1757 (50  $\mu$ M) (mGlu5 specific), the group II agonists DCGIV (500 nM) and NAAG (50  $\mu$ M) (mGlu3 specific), the group III agonists  $\alpha$ AP4 (100  $\mu$ M) and RSPPG (100  $\mu$ M), or the group III antagonist MAP4 (500  $\mu$ M). The concentrations used were adapted from previous studies of mGluR agonists and antagonists on microglia (Taylor et al., 2002; 2003; 2005; Pinteaux-Jones et al., 2008). No agonists or antagonists induced iNOS expression in BV-2 cells after 24 hours *in vitro* (Figure 6.3.1). An experiment using all the agonists and antagonists at double the concentrations normally used was also performed, to ensure that iNOS expression was not induced at higher concentrations (Figure 6.3.1 B).

This line of experiments was based on an interesting group of results obtained from the incubation of BV-2 cells with 1 mg/ml FV in combination with the mGluR agonists and antagonists for 24 hours. It was shown that when FV was applied to BV-2s in combination with the group III mGluR antagonist MAP4 (500  $\mu$ M), iNOS expression was strongly upregulated (Figure 6.3.2 A). This suggested that MAP4 can enhance FV induced toxicity in BV-2s and was in agreement with evidence for a neuroprotective role for the group III mGluRs (Taylor et al., 2003). Additionally, combination of FV with the group II mGluR agonists DCGIV (500 nM) or NAAG (50  $\mu$ M), upregulated iNOS expression, pointing towards a mechanism that could

enhance FV induced toxicity in BV-2s (Figure 6.3.2 A). This agreed with evidence about group II mGluR induced neurotoxicity to microglia (Taylor et al., 2005).

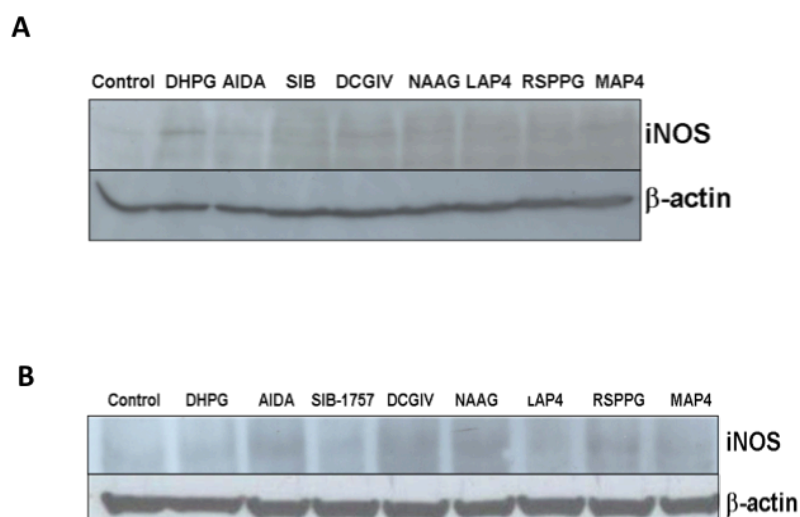
Due to the large amount of primary microglia needed to collect substantial cytoplasmic protein to run western blots and due to limitations on primary microglial availability, based on the FV activated BV-2 cell observations, only the mGluR agonists DCGIV and NAAG, and the antagonist MAP4 were studied in primary microglia. The results obtained following activation of primary microglia with 1 mg/ml FV and the agonists and antagonists selected, showed that the group II agonist NAAG could significantly increase iNOS expression in primary microglia (Figure 6.3.2 B). The densitometry results did not reveal statistical significance between FV activated microglia and FV with DCGIV, or MAP4 conditioned samples.

Repeating the same experiment with LPS in BV-2 cells, 1 µg/ml LPS was applied to BV-2s in combination with the group I agonist DHPG (100 µM), the group I antagonists AIDA (250 µM) and SIB-1757 (50 µM) (mGlu5 specific), the group II agonists DCGIV (500 nM) and NAAG (50 µM) (mGlu3 specific), the group III agonist L-AP4 (100 µM), or the group III antagonist MAP4 (500 µM). The results were variable so no safe conclusions could be reached from this single experiment (Figure 6.3.3 A). In primary microglia the results obtained showed that none of the group II agonists DCGIV or NAAG or the group III antagonist MAP4, were able to significantly increase iNOS expression (Figure 6.3.3 B).

Incubation of BV-2 cells with 2.5 mg/ml FG and the group I agonist DHPG resulted in slight increase in iNOS expression after 24 hours *in vitro* (Figure 6.3.4 A). In primary microglia, the group II agonists DCGIV and NAAG and the group III antagonist MAP4 were tested in combination with FG activation, based on the results acquired from the experiments where microglia were activated by FV (Figure 6.3.2). In primary microglia DCGIV incubation indicated a decrease rather than an increase in FG induced iNOS expression. MAP4 increased iNOS expression (Figure 6.3.4 B). This experiment was only performed once due to limitations in primary microglial numbers, so it should be repeated before conclusions are drawn.

Finally, 1 mg/ml FN was used to activate BV-2s and assess the effect of mGluR agonists and antagonists on iNOS expression in BV-2s. No agonists or antagonists altered the levels of iNOS expressed by BV-2s following 24 hour incubation with 1

mg/ml FN (figure 6.3.5 A). The same was true for primary microglia that were activated with 1 mg/ml FN in combination with the group II agonists DCGIV and NAAG and the group III antagonist MAP4. No significant alterations in iNOS levels were observed (Figure 6.3.5 B).



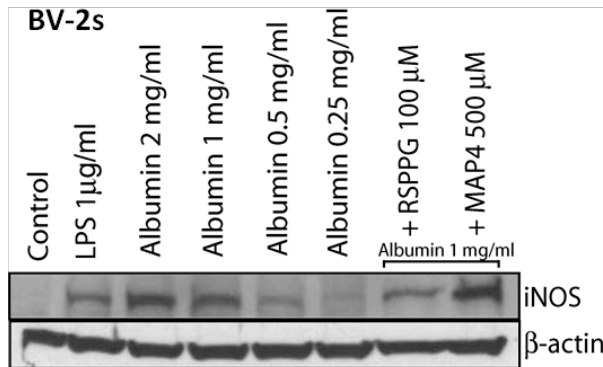
**Figure 6.3.1 mGluRs did not induce iNOS expression in BV-2 cells**

A. Representative western blot of iNOS expression in BV-2s after incubation with mGluR agonists or antagonists for 24 hours. The agonists and antagonists used were 100  $\mu$ M DHPG, 250  $\mu$ M AIDA, 50  $\mu$ M SIB-1757, 500 nM DCGIV, 200  $\mu$ M APICA, 50  $\mu$ M NAAG, 100  $\mu$ M  $\text{LAP4}$ , 100  $\mu$ M RSPPG or 500  $\mu$ M MAP4. No agonists or antagonists induced iNOS expression. This blot is representative of three different experiments. B. Western blot of iNOS expression in BV-2s after incubation with mGluR agonists or antagonists for 24 hours using double concentrations of agonists and antagonists. The concentrations used were 200  $\mu$ M DHPG, 500  $\mu$ M AIDA, 100  $\mu$ M SIB-1757, 1  $\mu$ M DCGIV, 100  $\mu$ M NAAG, 200  $\mu$ M  $\text{LAP4}$ , 200  $\mu$ M RSPPG or 1 mM MAP4. No agonists or antagonists induced iNOS expression.  $\beta$ -actin was used as a loading control.

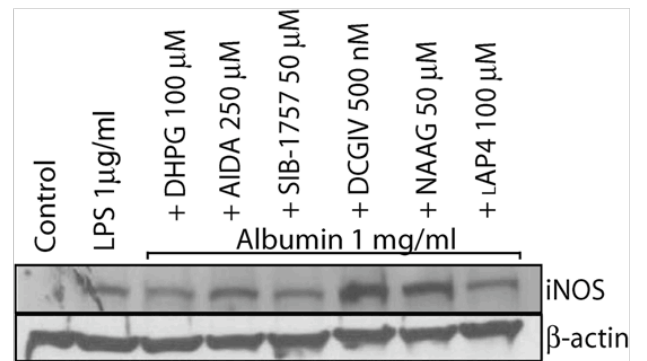
**Figure 6.3.2 mGluR group II agonists DCGIV and NAAG or group III antagonist MAP4 exacerbated iNOS expression in BV-2 cells exposed to FV albumin**

A. Representative western blots (i,ii) and densitometric analysis (iii) of iNOS expression of BV-2 cells exposed to (i) (0.25-2) mg/ml FV alone or in combination with 100 mM RSPPG or 500 mM MAP4, and (ii) 1 mg/ml FV in combination with 100 mM DHPG, 250 mM AIDA, 50 mM SIB-1757, 500 nM DCGIV, 50 mM NAAG or 100 mM MAP4 for 24 hours. Group III mGluR antagonist MAP4 enhanced FV induced iNOS expression. Group II mGluR agonists DCGIV and NAAG (mGlu3 specific) also enhanced FV induced iNOS expression. LPS 1 µg/ml was used as an internal positive control. The blots represent experiments repeated in triplicate. B. Representative western blot (i) and densitometric analysis (ii) of iNOS expression of primary microglia exposed to 1 mg/ml FV alone or in combination with 500 nM DCGIV, 50 mM NAAG or 500 mM MAP4 for 24 hours. Group II mGluR3 specific agonist NAAG significantly enhanced FV induced iNOS expression. Commercially available mouse macrophage LPS/IFN $\gamma$  cell lysate (BD Transduction 611473) was used as positive control.  $\beta$ -actin was used as a loading control. The experiments with primary microglia were repeated in duplicate. One way ANOVA with Tukey's post test was performed. Levels of significance were ns  $p>0.05$ , \*  $p<0.05$ , \*\*  $p<0.01$ , \*\*\*  $p<0.001$ .

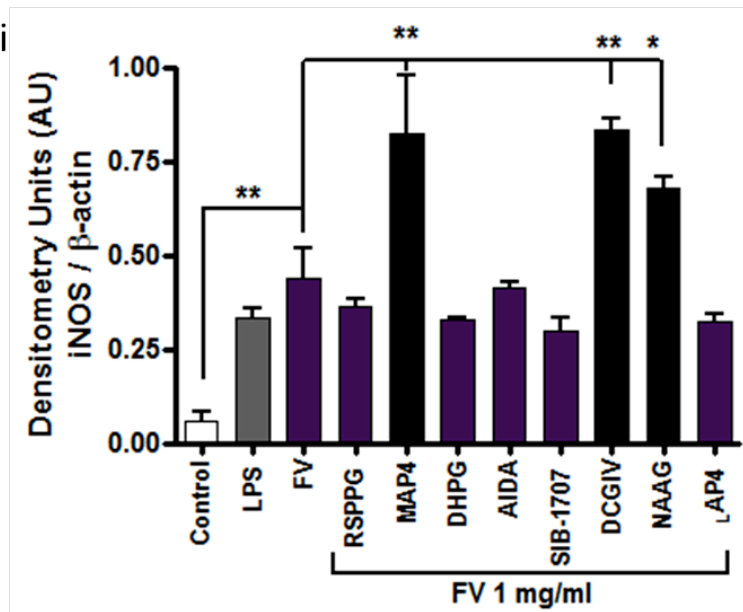
Ai



Aii

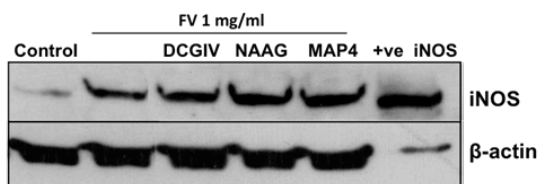


Aiii

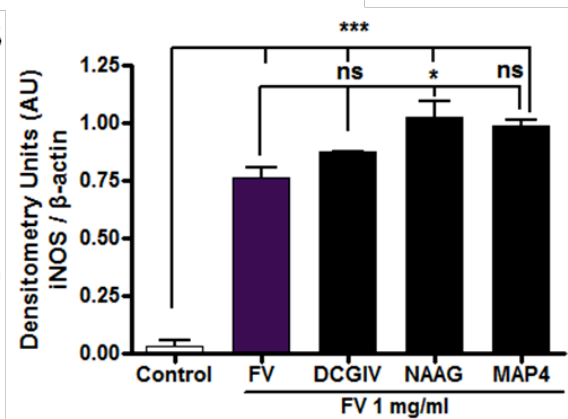


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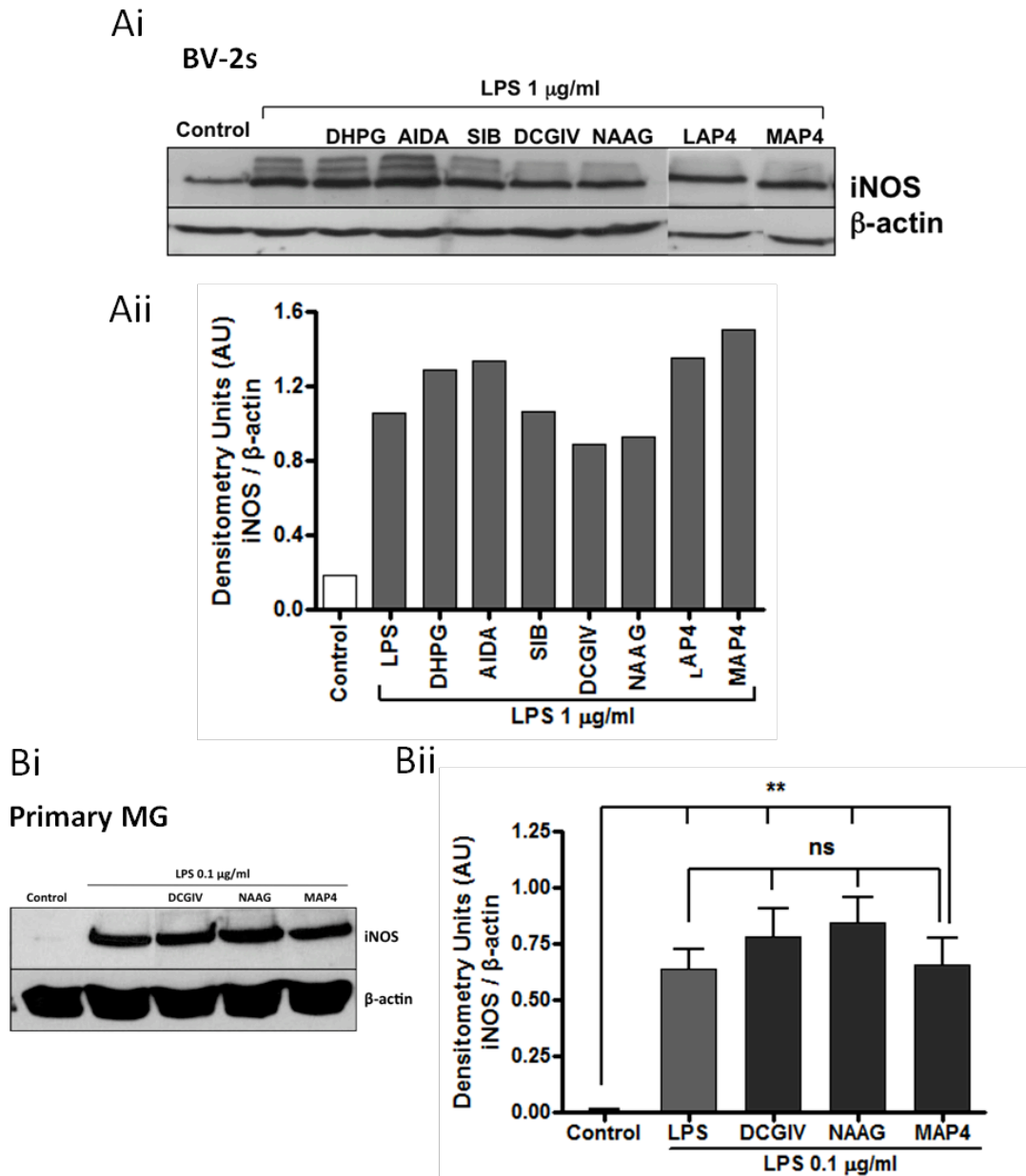
**Primary MG**



B

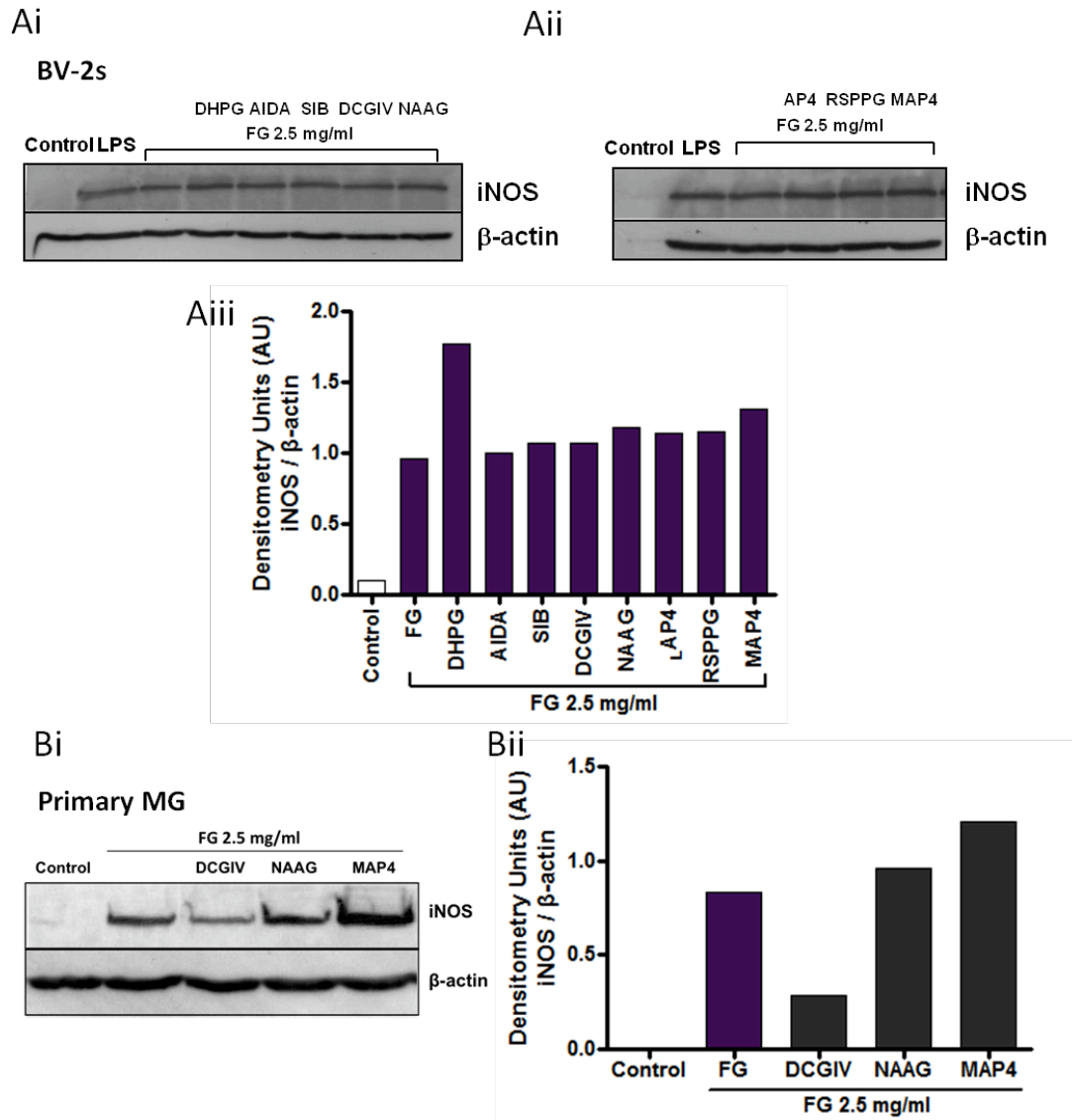






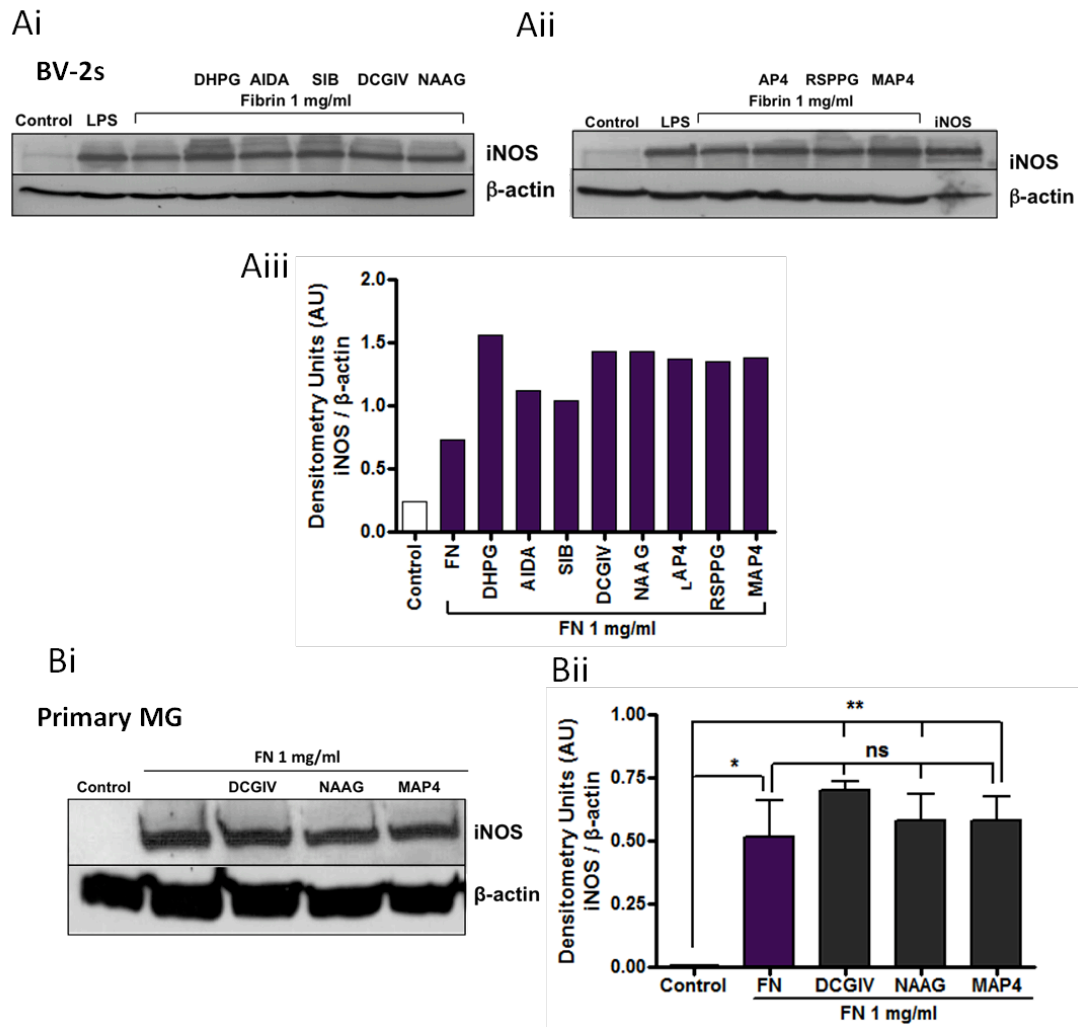
**Figure 6.3.3 mGluR manipulation did not alter iNOS expression in LPS activated microglia**

A. Western blot (i) and densitometric analysis (ii) of iNOS expression of BV-2 cells exposed to 1  $\mu\text{g/ml}$  LPS alone or in combination with 100 mM DHPG, 250 mM AIDA, 50 mM SIB-1757, 500 nM DCGIV, 50 mM NAAG, 100 mM  $\text{LAP4}$  or 500 mM MAP4 for 24 hours. B. Representative western blot (i) and densitometric analysis (ii) of iNOS expression of primary microglia exposed to 0.1  $\mu\text{g/ml}$  LPS alone or in combination with 500 nM DCGIV, 50 mM NAAG or 500 mM MAP4 for 24 hours. Group II mGluR agonists DCGIV and NAAG (mGlu3 specific) enhanced LPS induced iNOS expression but not significantly.  $\beta$ -actin was used as a loading control. The experiments were repeated in triplicate. One way ANOVA with Tukey's post test was performed. Levels of significance were ns  $p > 0.05$ , \*\*  $p < 0.01$ .



**Figure 6.3.4 mGluR manipulation of iNOS expression in fibrinogen activated microglia**

A. Western blots (i,ii) and densitometric analysis (iii) of iNOS expression of BV-2 cells exposed to (i) 2.5 mg/ml FG alone or in combination with 100 mM DHPG, 250 mM AIDA, 50 mM SIB-1757, 500 nM DCGIV, or NAAG, and (ii) 50 mM 100 mM  $\alpha$ AP4 100 mM RSPPG or 500 mM MAP4 for 24 hours. Group I mGluR agonist DHPG enhanced FG induced iNOS expression in BV-2s. LPS 1  $\mu$ g/ml was used as an internal positive control. B. Representative western blot (i) and densitometric analysis (ii) of iNOS expression of primary microglia exposed to 2.5 mg/ml FG alone or in combination with 500 nM DCGIV, 50 mM NAAG or 500 mM MAP4 for 24 hours. Group II mGluR agonist DCGIV reduced FG induced iNOS expression, while group III antagonist MAP4 increased FG induced iNOS expression.  $\beta$ -actin was used as a loading control.



**Figure 6.3.5 mGluR manipulation had no effect on iNOS expression in fibrin activated microglia**

A. Western blots (i,ii) and densitometric analysis (iii) of iNOS expression of BV-2 cells exposed to (i) 1 mg/ml FN alone or in combination with 100 mM DHPG, 250 mM AIDA, 50 mM SIB-1757, 500 nM DCGIV, or NAAG, and (ii) 50 mM 100 mM  $\Delta$ AP4 100 mM RSPPG or 500 mM MAP4 for 24 hours. LPS 1  $\mu$ g/ml was used as an internal positive control. Commercially available mouse macrophage LPS/IFN $\gamma$  cell lysate (BD Transduction 611473) was used as positive control. B. Representative western blot (i) and densitometric analysis (ii) of iNOS expression of primary microglia exposed to 1 mg/ml FN alone or in combination with 500 nM DCGIV, 50 mM NAAG or 500 mM MAP4 for 24 hours. None of the three agonists and antagonists used altered the levels of iNOS expression significantly.  $\beta$ -actin was used as a loading control. The experiment was repeated in triplicate. One way ANOVA with Tukey's post test was performed. Levels of significance were ns  $p > 0.05$ , \*  $p < 0.05$ , \*\*  $p < 0.01$ .

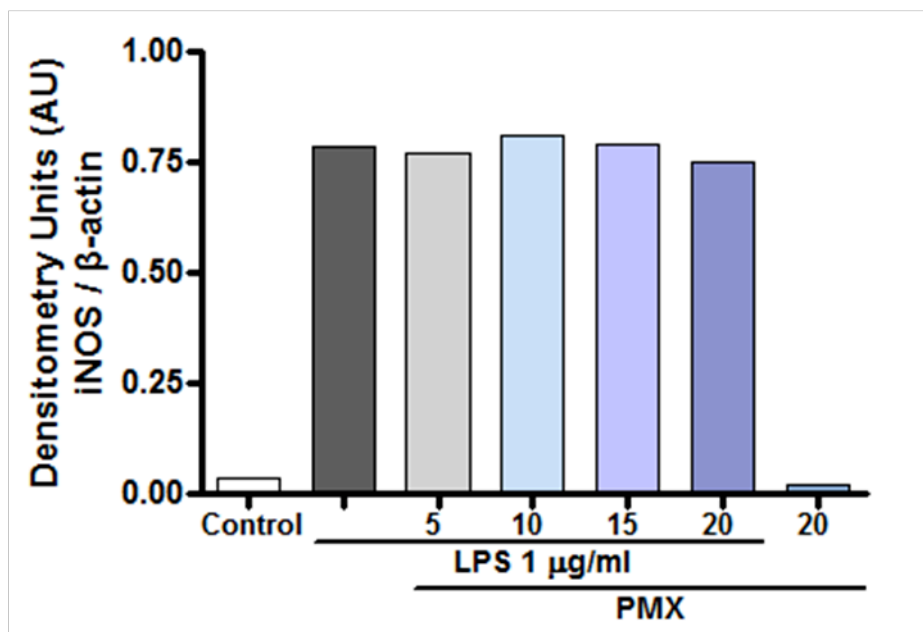
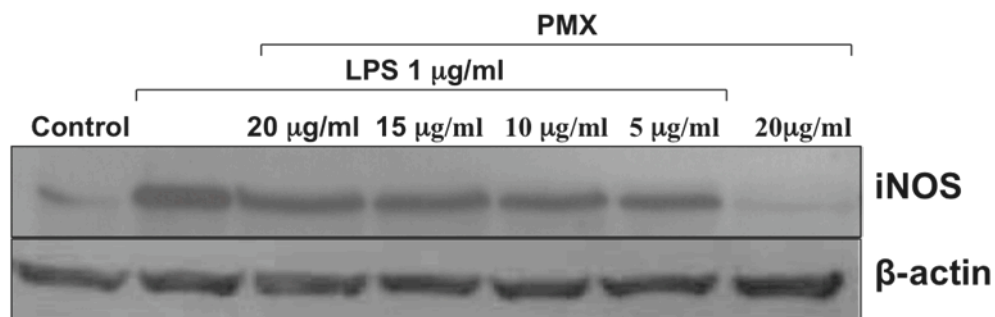
## 6.4 Investigation of BBP sample contamination by endotoxin

To investigate possible endotoxin contamination of the BBP solutions used in this study (Weinstein et al., 2008), an experimental plan was set to examine endotoxin contamination of the BBP solutions used on BV-2 cells.

Polymyxin B (PMX) is an LPS inhibitor that binds to the biologically active portion of LPS and potentially abrogates inflammatory responses (Inada, 1980; Dubor et al., 1986). Direct administration (data not shown), 24 hour preincubation of 1 µg/ml LPS activated BV-2s with (5-20) mg/ml PMX (Weinstein et al., 2008; Figure 6.4.1), or incubation of the LPS solution directly with PMX (data not shown) did not result in reduction in iNOS expression as observed with western blotting. Thus, since PMX did not block iNOS expression, it was not possible to study whether iNOS expression was endotoxin rather than BBPs induced in BV-2s.

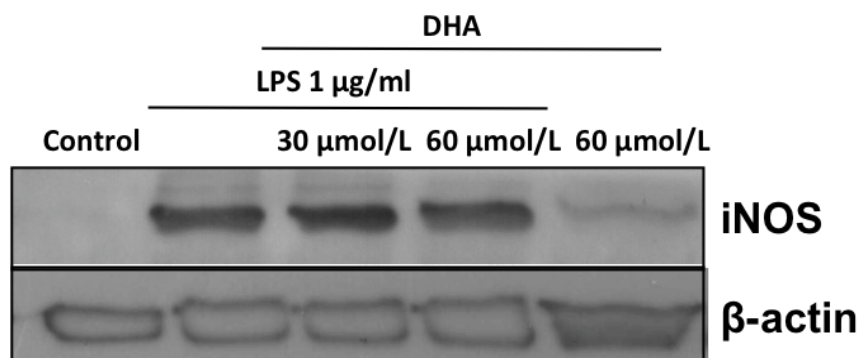
It has been suggested that docosahexaenoic acid (DHA), a fatty acid highly incorporated in the brain, is a potent immunomodulator that targets LPS receptor locations and therefore reduces LPS action on microglia, as shown by inhibition of the NF-κB pathway and reduction of TNF-α production (De Smedt-Peyrusse et al., 2008). DHA was thus used to block iNOS expression in LPS activated BV-2 cells. DHA was unable to block iNOS expression at 30 µmol/L (De Smedt-Peyrusse et al., 2008), or at 60 µmol/L (Figure 6.4.2). At higher concentrations DHA was toxic to BV-2s.

To measure the endotoxin content of the BBP solutions used in this study, an endotoxin quantification kit (LAL, Associates of Cape Cod Incorporated) was used. The principle of the method was discussed in 2.9. The results obtained by the LAL kit were not reproducible and revealed high amounts of endotoxin in all samples tested. Even filtered, UV irradiated distilled water, which was routinely used for culture of control cell cultures that did not exhibit signs of activation (i.e. iNOS expression), was shown to contain high amounts of endotoxin. FV, as well as FG and FN revealed high levels of endotoxin, but the results were similar for commercially available endotoxin free bovine albumin (Sigma, A6414). The LAL endotoxin test results are summarised in Table 6.4.1. These findings will be compared with other approaches used to identify whether the BBP responses of microglia are endotoxin mediated (see 6.6).



**Figure 6.4.1 PMX did not block iNOS expression in LPS activated microglia**

Representative western blot and densitometric analysis of iNOS expression of 1 μg/ml LPS activated BV-2 cells preincubated with (5-20) μg/ml of the LPS inhibitor PMX for 24 hours. PMX was unable to block iNOS expression in BV-2 cells. PMX alone did not induce iNOS expression. β-actin was used as a loading control.



**Figure 6.4.2 DHA did not block iNOS expression in LPS activated microglia**

Representative western blot and densitometric analysis of iNOS expression of 1 μg/ml LPS activated BV-2 cells preincubated with (30-60) μmol/L of the LPS inhibitor DHA for 24 hours. DHA was unable to block iNOS expression in BV-2 cells. DHA alone did not induce iNOS expression. At higher concentrations DHA was toxic to the cells. β-actin was used as a loading control. The experiment was repeated in duplicate.

Experiment	Sample	#1 EU/ml	#2 EU/ml
<b>1</b>	Negative control	0.075	
	LPS 5 µg/ml	0.166	
	FV Albumin 50 mg/ml	0.197	
	FV Albumin 20 mg/ml	0.183	
	FV Albumin 1 mg/ml	0.152	
	FV Albumin 0.5 mg/ml	0.154	
	FV Albumin 0.25 mg/ml	0.123	
<b>2</b>	Negative control	0.021	
	LPS 1 µg/ml	0.268	
	FV Albumin 12 mg/ml	0.292	0.261
	FV Albumin 2 mg/ml	0.262	0.294
	FV Albumin 1 mg/ml	0.282	0.278
<b>3</b>	Negative control	0.007	0
	LPS 1 µg/ml	0.941	0.853
	Distilled water	0.961	
<b>4</b>	Negative control	0	
	Distilled water	0.756	
	LPS 0.5 µg/ml	0.996	
	LPS 0.25 µg/ml	0.914	
	FV Albumin 12 mg/ml	0.945	0.967
	FV Albumin 2 mg/ml	0.961	1.002
	FV Albumin 1 mg/ml	0.941	1.010
	Fibrinogen 2.5 mg/ml	0.966	0.996
	Fibrin 1 mg/ml	1.005	0.978
	Filtered/ UV water		0.960
	Endotoxin free Albumin 1 mg/ml		0.701

**Table 6.4.1 LAL Pyrochrome endotoxin assay for the determination of endotoxin in BBP samples**

The LAL Pyrochrome endotoxin assay was used to determine the endotoxin content of BBP solutions used to activate microglia. All results are expressed in endotoxin units / ml (EU/ml). Four different experiments were performed and some of the samples were tested in duplicate. All samples tested, as well as UV irradiated, filtered distilled water, were shown to contain high endotoxin levels, similar to those of LPS. The assay was easily saturated as can be seen from LPS or FV albumin titration measurements and was not always reproducible.

## 6.5 Microglial BBP receptors

To further investigate whether microglial responses to FV, FG or FN were mediated by contaminating LPS residues, it was attempted to identify receptors to which the BBPs could bind to and study whether those receptors were identified as LPS receptors as well. This could help elucidate the issue of whether different BBPs activate microglia through distinct or identical to LPS pathways.

LPS activates microglia by binding to toll-like receptors, predominantly toll-like receptor 4 (TLR4), and initiates the microglial immune response (reviewed by Olson & Miller, 2004). LPS binding to TLR4 triggers the dimerisation of the receptor and the formation of a tetrameric structure consisting of two TLR4 receptors and two myeloid differentiation factor 2 (MD-2) membrane proteins, which are necessary for receptor dimerisation (Park et al., 2009). It was attempted to block LPS induced iNOS activation by using an anti-TLR4 antibody aimed for inhibitory studies (Santa Cruz, sc-13591 L). Anti-TLR4 at 3 µg/ml successfully reduced iNOS expression in BV-2 cells activated with 0.5 µg/ml LPS. Since anti-TLR4 was able to attenuate LPS induced microglial activation, its effect on BV-2 cells activated with FV, FG or FN was studied. Anti-TLR4 did not reduce iNOS expression in BV-2 cells activated with 1 mg/ml FV, 2.5 mg/ml FG or 1 mg/ml FN, indicating that microglial activation by BBPs is not dependent on the TLR4 receptor (Figure 6.5.1). Interestingly, anti-TLR4 incubation increased iNOS expression in FV and FG activated microglia, indicating that blockage of the TLR4 receptor-induced pathway in microglia activated with FV or FG initiates a mechanism able to trigger iNOS expression. It is possible that this pathway is triggered by TLR4 receptor ligation, where interaction of two TLR4 receptor subunits on the BV-2 membrane may be enhanced by the presence of the blocking antibody, which with its two antigen binding sites can bind to two TLR4 receptors and bring them in close proximity, thus resulting in their dimerisation, followed by activation of intracellular pathways.

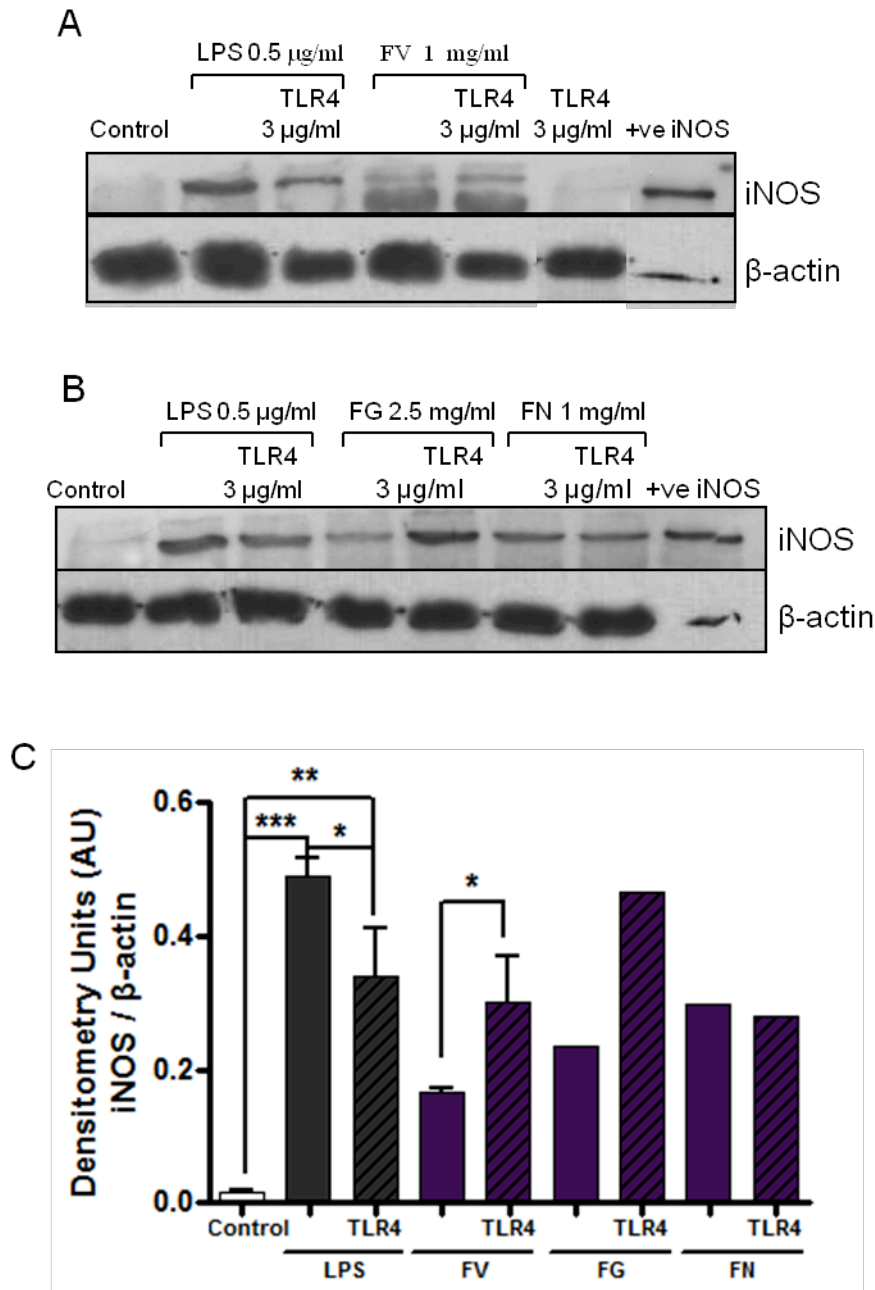
FG has been shown to bind to the Mac-1 integrin receptor (CD11b) present on microglia (Adams et al., 2007). Multiple Mac-1 recognition sites have been described for FG (Lishko et al., 2004). In this study activation of BV-2 cells by binding of FG and FN to the CD11b receptor on microglia was attempted. Although restricted to just FG



and not FN as previously shown (Adams et al., 2007), anti-CD11b was able to attenuate FG induced iNOS expression in BV-2s. A slight increase in FV induced iNOS expression in the presence of anti-CD11b was observed (Figure 6.5.2 A). TNF- $\alpha$  released from BV-2s activated in the presence of anti-CD11b was also measured (Figure 6.5.2 B). The kit used (Quantikine Rat TNF- $\alpha$ , R&D Systems, Oxford, UK) was rat specific so with BV-2 cells (mouse origin) the TNF- $\alpha$  signal was weak. Still some observations could be made. Addition of anti-CD11b to LPS activated BV-2s increased TNF- $\alpha$  release. FG induced release of TNF- $\alpha$  was also increased. This increase in TNF- $\alpha$  release could be explained by ligation of the CD-11b receptor in the presence of the blocking antibody, which with its two antigen binding sites could bind to two CD-11b receptor domains and bring them in close proximity, thus resulting in their dimerisation, followed by activation of intracellular pathways leading to TNF- $\alpha$  release.

In the case of primary microglia, activation by binding of FG and FN to the CD11b receptor on microglia was attempted. In agreement with the BV-2 data, the anti-CD11b antibody reduced FG but not FN induced iNOS expression (Figure 6.5.3). Additionally, FV induced iNOS expression in the presence of anti-CD11b was upregulated (Figure 6.5.3 A). TNF- $\alpha$  released from primary microglia activated in the presence of anti-CD11b was also measured (Figure 6.5.3 B). Addition of anti-CD11b to FG and FN activated microglia increased TNF- $\alpha$  release. LPS and FV induced iNOS expression was not increased, but the assay was saturated at high levels of TNF- $\alpha$ , so increase in TNF- $\alpha$  release could not be measured further.

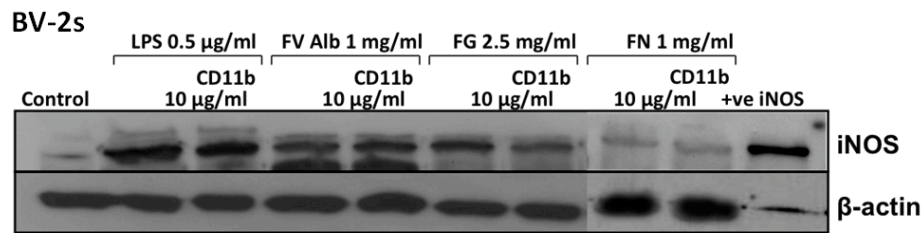
These experiments show that anti-CD11b is able to attenuate microglial iNOS expression following activation with FG but not FN. At the same time TNF- $\alpha$  release is increased in both FG and FN activated primary microglia, and it is postulated that this may be due to receptor ligation in the presence of the specific anti-CD-11b antibody. As in the case of TLR4, CD11b blockage upregulated other microglial activating pathways, leading to iNOS expression increase (FV induced) or TNF- $\alpha$  release. The receptors required and the pathways activated by LPS or BBPs will be summarised in this chapter's discussion.



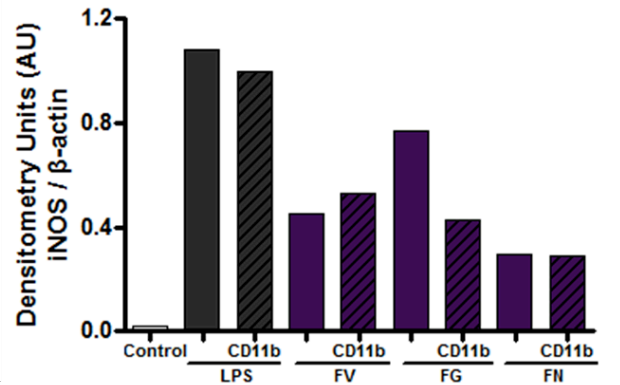
**Figure 6.5.1 TLR4 antibody attenuated LPS induced iNOS expression in BV-2s**

A, B. Western blots and densitometric analysis (C) of iNOS expression of BV-2 cells exposed to 0.5  $\mu$ g/ml LPS, 1 mg/ml FV, 2.5 mg/ml FG or 1 mg/ml FN for 24 hours alone or after preincubation with 3  $\mu$ g/ml LPS receptor antibody TLR4 for 24 hours. Anti-TLR4 attenuated LPS induced iNOS expression. FV, FG or FN induced iNOS expression was not reduced by anti-TLR4, indicating that microglial activation by those BBPs is not dependent on the TLR4 receptor. Interestingly, anti-TLR4 incubation increased iNOS expression in FV and FG activated microglia.  $\beta$ -actin was used as a loading control. The LPS and FV experiments were repeated in duplicate. The FG and FN experiment was only performed once. Treated groups were compared with the control using one way ANOVA with Tukey's post test. Levels of significance were \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

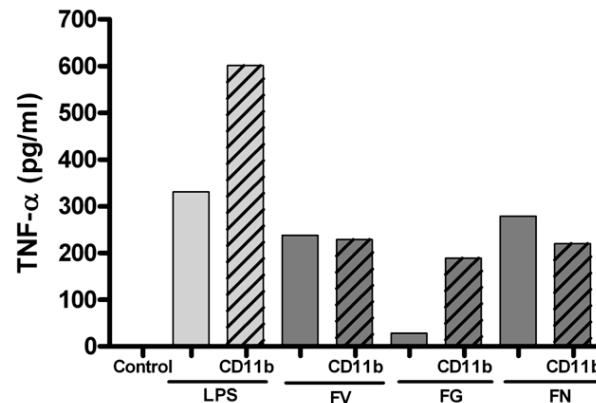
Ai



Aii



B

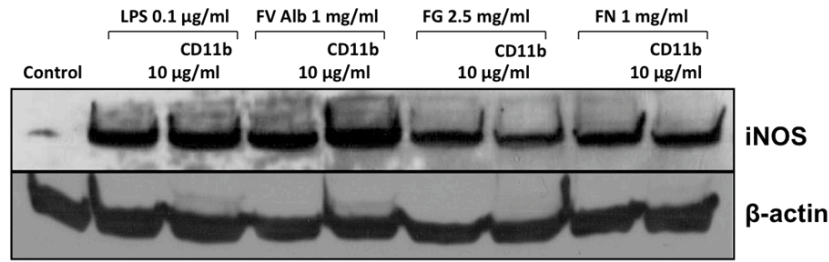


**Figure 6.5.2 The effect of the CD11b receptor antibody on differentially activated BV-2s**

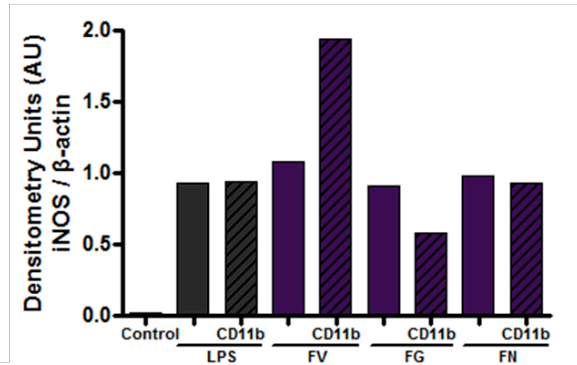
A. Western blot (i) and densitometric analysis (ii) of iNOS expression of BV-2 cells exposed to 0.5  $\mu\text{g/ml}$  LPS, 1 mg/ml FV, 2.5 mg/ml FG or 1 mg/ml FN for 24 hours alone or after preincubation with 10  $\mu\text{g/ml}$  of the FG receptor antibody CD11b for 24 hours. CD11b attenuated FG induced iNOS expression. LPS, FV or FN induced iNOS expression was not reduced by CD11b, indicating that microglial activation by those proteins was CD11b independent. A slight increase in FV induced iNOS in the presence of CD11b was observed. Commercially available mouse macrophage LPS/IFN $\gamma$  cell lysate (BD Transduction 611473) was used as positive control.  $\beta$ -actin was used as a loading control. B. Medium from the experiment on BV-2s was collected and TNF- $\alpha$  release was measured with an ELISA kit. The kit used (Quantikine Rat TNF- $\alpha$ , R&D Systems, Oxford, UK) was rat specific so with BV-2 cells (mouse origin) the TNF- $\alpha$  signal was weak. Addition of CD11b to LPS activated microglia increased TNF- $\alpha$  release. FG induced release of TNF- $\alpha$  was also increased.

Ai

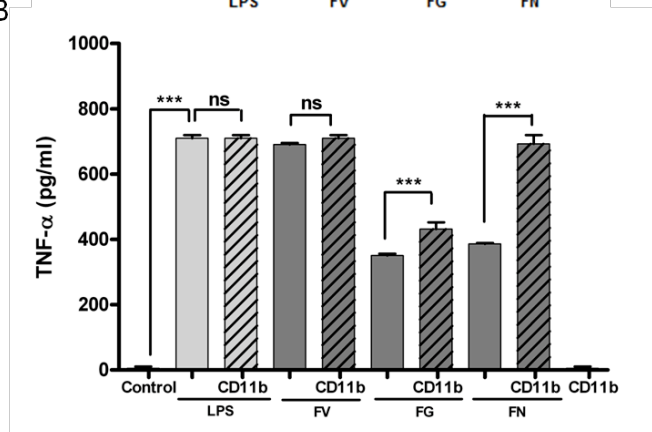
### Primary MG



Aii



B

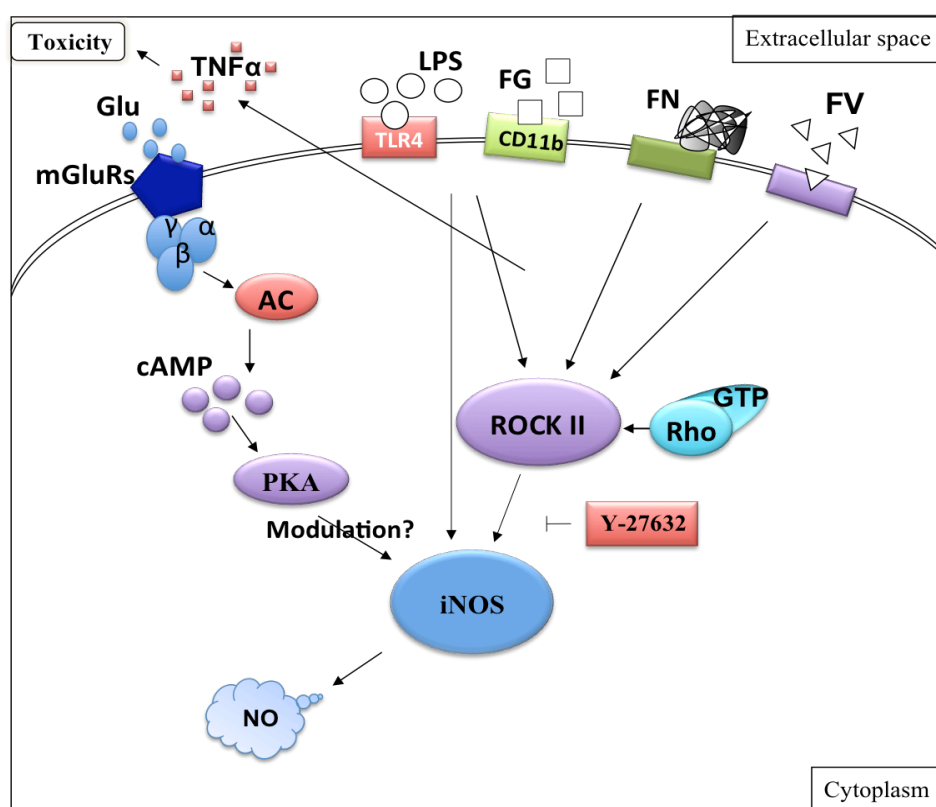


**Figure 6.5.3 The effect of the CD11b receptor antibody on differentially activated primary microglia**

A. Western blot (i) and densitometric analysis (ii) of iNOS expression of primary microglia exposed to 0.5  $\mu\text{g/ml}$  LPS, 1 mg/ml FV, 2.5 mg/ml FG or 1 mg/ml FN for 24 hours alone or after preincubation with 10  $\mu\text{g/ml}$  of the FG receptor antibody CD11b for 24 hours. CD11b attenuated FG induced iNOS expression. LPS, FV or FN induced iNOS expression was not reduced by CD11b, indicating that microglial activation by those proteins was CD11b independent. Additionally, CD11b blockade increase iNOS expression by FV activated microglia.  $\beta$ -actin was used as a loading control. B. Medium from the experiment on microglia was collected and TNF- $\alpha$  release was measured with an ELISA kit. Addition of CD11b to FG and FN activated microglia increased TNF- $\alpha$  release. LPS and FV induced iNOS expression was not increased, but the assay was saturated at high levels of TNF- $\alpha$  release. The experiment was repeated in duplicate. One way ANOVA with Tukey's post test was performed. Levels of significance were ns  $p>0.05$  and \*\*\*  $p<0.001$ .

## 6.6 Discussion

This chapter was focused on studying differential microglial activation by LPS or BBPs, involvement of potential receptors in BBP mediated microglial activation, intracellular pathways mobilised following activation and the potential of modulating microglial activation by mGluRs. The findings of this chapter are summarised in figure 6.6.1.



**Figure 6.6.1 Proposed iNOS activating pathways in microglia**

Schematic representation of the proposed pathways activated following microglial activation with LPS (lipopolysaccharide), FV (FV albumin), FG (fibrinogen) or FN (fibrin). LPS and FG can induce iNOS (inducible nitric oxide synthase) expression by a ROCK (Rho-associated coiled coil-containing protein kinase) dependent and a ROCK independent pathway. FV and FN induce iNOS expression via the ROCK dependent pathway that can be blocked by the ROCK inhibitor Y-27632. TNF- $\alpha$  (tumour necrosis factor - $\alpha$ ) release occurs upstream of ROCK activation and can be upregulated if membrane receptors are blocked. Finally the group II and III mGluRs (metabotropic glutamate receptors) are implicated in iNOS upregulation following microglial activation primarily with FV. mGluRs could modulate iNOS expression via AC (adenyl cyclase) activation, cAMP (cyclic adenosine monophosphate) signalling and PKA (protein kinase A) phosphorylation.

### **6.6.1 Rho-ROCK mediated iNOS expression in microglia**

It was shown here that LPS, FV, FG or FN induced iNOS expression by microglia could be attenuated by incubation with the ROCK inhibitor Y-27632. In BV-2 cells Y-27632 potently blocked iNOS expression induced by LPS or BBPs (Figures 6.2.1, 6.2.2, 6.2.3, 6.2.4). In primary microglia FV and FN induced iNOS expression was potently downregulated by Y-27632 (Figures 6.2.2 and 6.2.4). In LPS or FG activated primary microglia though, Y-27632 could only partially downregulate iNOS expression (Figures 6.2.1 and 6.2.3). This partial ability of ROCK inhibition to block iNOS expression points towards an additional, ROCK-independent pathway for microglial iNOS expression (Figure 6.6.1). Microglial cells have been shown to release nitric oxide via a Rho and NF- $\kappa$ B, but not a ROCK dependent pathway (Hoffmann et al., 2008). It is thus possible that differential microglial activation might lead to iNOS expression via Rho mediated pathways that may or may not depend on ROCK activation, or by a completely distinct, Rho-ROCK independent pathway.

The Rho-ROCK pathway in macrophage lineage cells is implicated in actin regulation, process formation and migration (Allen et al., 1997), as well as in complement-mediated phagocytosis (Caron & Hall, 1998). RhoA or ROCK depletion was involved in cyclooxygenase-2 (COX-2) related anti-inflammatory processes in spinal cord lesions (Schwab et al., 2004). Cytoskeleton rearrangement and inflammatory processes, the two cellular functions with which Rho-ROCK has been associated, are linked by phagocytosis, a process that requires rapid actin rearrangement and is associated with external cell signalling (reviewed by Chimini & Chavrier, 2000). ROCK inhibition might thus be a promising target for modulating microglial activation, by regulating phagocytosis and attenuating microglial inflammatory responses and consequent neurotoxicity.

### **6.6.2 mGluR modulation of iNOS expression in microglia**

The group II mGluR agonists DCGIV and NAAG and the group III mGluR antagonist MAP4 induced a significant upregulation of iNOS expression following FV activation of BV-2 cells (Figure 6.3.2). The upregulation of iNOS in the presence of group II mGluR agonists was in agreement with evidence for a neurotoxic role for group II

mGluR receptors (Taylor et al., 2005). Blocking group II receptors could thus be a viable therapeutic strategy for attenuating microglial activation and ameliorating toxic effects on neurones and oligodendrocytes in MS. The upregulation of iNOS in the presence of the group III antagonist MAP4 agreed with data for a neuroprotective role for the group III mGluRs (Taylor et al., 2003). This suggests that group III blockade should be avoided when addressing chronic microglial activation, since it exacerbates activation. At the same time group III mGluR receptor activation could reduce sustained microglial activation and protect neurones and oligodendrocytes in chronic activation conditions, such as in progressive MS lesions. In an *in vitro* study, a group III agonist was able to reverse neuronal cell death induced by myelin activated microglia (Pinteaux-Jones et al., 2008).

The data obtained with FV activation of BV-2s were not successfully reproduced with primary microglia since no statistical significance was reached. A reason for this may be the fact that LPS or BBPs activate primary microglia more potently than the BV-2 cell line, so quantitative differences in protein expression are not easily distinguished. From these experiments though, it was demonstrated that mGluRs do have the ability to modulate iNOS expression, via pathways yet unidentified.

Modulation of microglial activation was demonstrated in the experiments where cells were activated with FV. FV is internalised by microglia (Figure 5.3.1.5), so it is possible that mGluR interference in iNOS upregulation may be triggered by phagocytosis. Even though there are no data supporting modulation of phagocytosis by mGluRs, it would be of interest to study intracellular relationships between mGluR related proteins such as PKA and proteins involved in the iNOS pathway. Finally, there is evidence that in dorsal root ganglion neurones expression of mGlu2 and mGlu3 is endogenously regulated by the NF- $\kappa$ B family of transcription factors (Chiechio et al., 2006). NF- $\kappa$ B is involved in iNOS expression (Pahan et al., 2001), so a link between iNOS and mGluR expression could be established based on this observation.

### **6.6.3 Is microglial BBP signalling endotoxin induced?**

Microglia recognise pathogen-derived ligands via binding of conserved pattern-recognition receptors, the toll-like receptors (TLRs). TLRs bind highly conserved

structural motifs such as PAMPs, which are typically essential for the survival of the respective pathogen, such as lipopolysaccharide (LPS) derived from gram-negative bacteria, which binds to TLR4 receptor. TLRs mediate microglial innate immunity but also link with the adaptive immune response (reviewed by Lehnardt, 2009). It was shown here that TLR4 receptor blockade attenuated iNOS expression in microglia (Figure 6.5.1). On the other hand, FV, FG or FN induced iNOS expression was not attenuated by blocking TLR4 ligation (Figure 6.5.1). In addition, blockage of TLR4 receptor in the presence of FV or FG upregulated iNOS expression in microglia, a result which suggests that the TLR4 blocking antibody may promote TLR4 dimerisation by bringing two molecules of the TLR-4/MD-2 complex (Park et al., 2009) in close proximity, thus resulting in cellular responses such as iNOS expression. Thus, even though the LAL endotoxin kit revealed high levels of endotoxin in the BBP solutions used (which was also the case for all samples tested, including UV irradiated, filtered distilled water, Table 6.4.1), the TLR4 experiments provided direct proof that BBPs do not signal via TLR4. Thus, the next step was to identify alternative receptors for BBP induced microglial activation.

FG and FN have been shown to bind to the  $\alpha$  chain of the integrin receptor Mac-1, CD11b (Adams et al., 2007). This was based on the well established concept that the integrin receptor subunit CD11b is the macrophage receptor for FG (Ugarova & Yakubenko, 2001; Lishko et al., 2002). In this study a decrease in iNOS expression following FG but not FN activation of microglia was observed by blocking the CD11b receptor (Figure 6.5.3). That was not the case for TNF- $\alpha$  release, which was upregulated after CD11b blockage in both FG and FN activated microglia (Figure 6.5.3). Again, this may result from the anti-CD11b antibody binding with two different CD11b receptors, promoting their dimerisation and possibly activating intracellular pathways resulting in TNF- $\alpha$  release. Also, even though there is data supporting CD11b activation as the primary event of FG mediated microglial activation, the possibility that there may be additional microglial receptors to which FG may bind should not be ruled out. For example, FG has been shown to bind to intercellular adhesion molecule-1 (ICAM-1) on endothelial cells, a molecule that signals through the Rho-GTPase pathway (Tsakadze et al., 2002). Furthermore, TLR2



receptor, which binds lipoproteins, peptidoglycans and polymeric molecules (Lehnardt, 2009), is a promising candidate for FG and FN ligation.

FV mediated microglial activation was questioned because high endotoxin was found in the commercially available reagent (Weinstein et al., 2008). A number of findings in relation to FV mediated microglial activation though point towards FV being a potent microglial activator. In microglia, albumin can induce an increase in cytoplasmic calcium, mediated via Src tyrosine kinases and phospholipase C, and leading to calcium-dependent proliferation (Hooper et al., 2005). It was also shown that microglial activation with lipid free albumin resulted in superoxide production *in vitro* (Si et al., 1997). LPS did not mimic the actions of FV in microglia. Furthermore in this study, except from the fact that FV microglial activation was not mediated by TLR4, the results obtained by FV activation of BV-2s, showing a significant upregulation of iNOS by group II mGluR agonists DCGIV and NAAG and group III mGluR antagonist MAP4 (Figure 6.3.2), were not replicated with LPS.

To conclude the discussion on whether BBP observed effects on microglia are endotoxin mediated, some more observations made in this study should be delineated. FV and FG only activate microglia when incubated in serum free medium, whilst LPS activates microglia even in medium containing 10% FBS. This may result from desensitisation of the FV and FG receptors when serum is present, while LPS receptors may not desensitise in the presence of serum. Furthermore, in chapter 5 a detailed comparison of the effect of LPS, FV, FG or FN activated microglia in oligodendrocyte lineage cell viability was made. It thus revealed many differences between the microglial activators (summarised in Table 5.5.1), as well as interesting similarities between LPS - FG or FV - FN, pointing towards membrane receptor mediated microglial activation by LPS and FG, and internalisation mediated microglial activation by FV and FN. In conclusion, even if there is an endotoxin component in the BBP reagents used when studying microglial incubation, there are plenty of data demonstrating differential action of LPS, FV, FG or FN on microglia.

#### **6.6.4 Conclusion**

In MS, BBPs are present in acute and chronic lesions (see 1.4.2.1). Differential microglial activation may involve distinct receptors and pathways, or may converge

to common pathways resulting in the expression of inflammatory cytokines and NO. Detailed knowledge of the pathways involved in microglial activation could provide a valuable tool for the development of drugs targeting microglial activation, since it would allow for the design of drugs that could preferentially block pathways leading to toxicity and preserve neuroprotective pathways. The rationale behind targeting mGluRs as modulatory targets is based on studies revealing their neurotoxic or neuroprotective effects *in vitro* (Taylor et al., 2003; Taylor et al., 2005; Pinteaux-Jones et al., 2008) or *in vivo* (Corti et al., 2007) and is complemented by the fact that mGluR stimulation or blockade in the CNS does not interfere with synaptic transmission (reviewed by Marek, 2004). Thus, as discussed in 1.5.3, if microglial mGluR manipulation could have a regulatory role in oligodendrocyte toxicity in MS, since mature oligodendrocytes only possess functional mGluR3 receptors (Luyt et al., 2006), therapeutic avenues targeting microglia without side effects on oligodendrocytes could be developed.

## **7. GENERAL DISCUSSION**

## 7.1 Microglial induced oligodendrocyte toxicity and protection in MS

The aim of this study was to investigate interactions between microglia and cells of the oligodendrocyte lineage, in an attempt to draw conclusions about the role of activated microglia present in acute or chronic MS lesions. Microglial activation in MS lesions is well documented in all disease stages, such as the newly defined preactive lesions (Gay et al., 1997; De Groot et al., 2001), the active, demyelinating lesions where microglia phagocytose debris and facilitate regeneration (Takahashi et al., 2005; Piccio et al., 2007; Nielsen et al., 2009), as well as in chronic neurodegenerative lesions where microglia are often located at the margins of demyelinated lesions (Peterson et al., 2002; Hill et al., 2004; Gray et al., 2008).

Factors that have been suggested to contribute to microglial activation in MS are bacterial toxins (Gay, 2007), viral components (Lipton et al., 2007), myelin debris (Williams et al., 1994; Pinteaux-Jones et al., 2008) and the small heat shock protein  $\alpha$ B-crystallin (Bhat & Sharma, 1999). This study was focused on microglial activation induced by BBPs. BBPs enter the brain following BBB disruption, a typical event in MS. In MS lesions, perivascular activation of microglia colocalises with areas of BBB disruption (reviewed by Minagar and Alexander, 2003). In accordance with previous data (Hooper et al., 2005; Adams et al., 2007; Hooper et al., 2009) it was shown that FV, FG and its polymer, FN caused microglial activation, as assessed by morphological changes, TNF- $\alpha$  release, iNOS expression and internalisation. Since elevated levels of these proteins, which are normally excluded from the brain, are able to activate microglia, they undoubtedly have an important role in microglial activation during all stages of MS. Furthermore, it was shown that BBPs do not act on microglia via the same receptors and pathways as LPS, they are thus able to differentially activate microglia and elicit distinct effects.

FG binds on the integrin CD11b receptor and activates microglia (Adams et al., 2007). FG activated microglia induced oligodendrocyte death when microglia and oligodendrocyte lineage cells were cultured together. On the contrary, when microglia were activated with FG and medium collected following a 24 hour activation was applied on oligodendrocytes, the effect was protective and

oligodendrocytes survived better. Thus, soluble, stable factors that are released from microglia may be protective to oligodendrocytes that are not in close proximity to microglia in MS lesions, whilst when microglia migrate near cells, additional signalling, possibly mediated by cell contact might trigger toxic cascades that promote phagocytosis and cell death. This microglial response may be triggered by degenerating oligodendrocytes expressing stress signals following upregulation of genes such as the inflammation related gene STAT6 (Zeis et al., 2009), or by merely the presence of exposed myelin. Microglia migrate to the sites of degeneration where they exacerbate toxicity and help clear up the lesion. At the same time, protective factors, such as IGF-1 (Pang et al., 2007) or IGF-2 (Nicholas et al., 2002) released from microglia may protect healthy oligodendrocytes or OPCs recruited to the site of injury to initiate remyelination after the inflammatory cascade of events has culminated. The action of FG on microglia resembled the results obtained following microglial activation with LPS, particularly when studying toxic effects on OPCs. LPS activated microglial conditioned medium was protective to OPCs, in contrast to findings by Pang et al., 2000, whilst when the two cell populations were in contact, OPC death was induced (Zajicek et al., 1992, Lehnardt et al. 2002, Li et al., 2008). LPS activated microglia were toxic to OPCs in a TNF- $\alpha$  dependent manner. It can be proposed that local high concentration of TNF- $\alpha$  is required for toxicity, hence microglia are toxic to OPCs in co-culture in close proximity, less toxic when cultured on inserts above OPCs, whilst conditioned medium is protective. An additional factor that may be implicated in those processes is Fas ligand, which was shown to induce neurotoxicity in concert with TNF- $\alpha$  (Taylor et al., 2005). Mature oligodendrocytes were also sensitive to LPS activated microglia, in agreement with Domercq et al., 2007 and in contrast to Miller et al., 2007 that found LPS to be protective in co-cultures. Oligodendrocyte death was shown to be TNF- $\alpha$  dependent. Mature oligodendrocytes responded similarly to FG activated and LPS activated microglia. The common effects of LPS or FG activated microglia imply that they both activate microglia via similar pathways in a receptor-mediated manner.

FV and FN also exhibited distinct functions with interesting similarities. Both proteins were directly toxic to OPCs but their toxicity was ameliorated when microglia were added to the OPC cultures. This indicated that microglia could clear

FV or FN from the area around OPCs and thus protect them from the toxic effects of those proteins. FV was internalised by microglia, a finding confirming this hypothesis. Commercially available FITC conjugated FV albumin allowed for such an experiment. Unfortunately FITC conjugated fibrin was not available to allow direct confirmation of this hypothesis with FN. In contrast to FV induced OPC death, FN induced OPC death was potently reversed when the Rho-ROCK pathway was inhibited. Since ROCK inhibition has already been proposed as a therapeutic strategy in MS due to its anti-inflammatory properties (Honing et al., 2004; Paintlia et al., 2005; Miron et al., 2007) its role in promoting OPC survival following exposure to FN points toward additional beneficial effects to cells other than macrophages. The mechanism by which ROCK inhibition protects OPCs remains to be elucidated.

## 7.2 Fibrin contribution to remyelination failure in MS

FN prevented OPC maturation into myelinating oligodendrocytes and preserved them in a bipolar, precursor stage. This finding implicated FN in MS pathology and was particularly obvious when OPCs were cultured in co-culture with microglia, where microglia prevented FN induced OPC death. In acute MS lesions, where there is BBB disruption (Claudio et al., 1995; Wakefield et al., 1994), the presence of FN may have adverse effects for disease progression by contributing to the well documented presence of quiescent or proliferating, but not differentiating OPCs in demyelinated lesions (Wolswijk, 1998; Chang et al., 2000; Solanky et al., 2001; Reynolds et al., 2002; Kuhlmann et al., 2008). This is complimented by the study in peripheral nerve injury in mice showing that plasminogen activator enhanced nerve regeneration and remyelination after injury (Song et al. 2006). It was also shown that FN regulated Schwann cell differentiation and inhibited remyelination in peripheral nerve injury (Akassoglou et al. 2002). Other molecules present in the extracellular space following brain injury or demyelination have also been implicated in OPC differentiation inhibition. As analytically discussed in 5.6.2, hyaluronan (Back et al., 2005), myelin (Robinson & Miller, 1999; Kotter et al., 2006) and PSA-NCAM (Charles et al., 2002) are all molecules implicated in OPC differentiation inhibition. Those molecules are high molecular weight polymers, or in the case of myelin lipidic membrane structures. This suggests that there may be a common mechanism by which these high molecular weight molecules can act upon OPCs and preserve them in an immature, proliferating stage. This finding has implications for disease pathology in MS and contributes to explain remyelination failure. Strategies that could remove FN from MS lesions or pharmacologically inhibit the intracellular pathways responsible for differentiation arrest could prove beneficial for remyelination enhancement and recovery in MS.

### 7.3 Suggestions for future work

This study yielded an interesting amount of data on interactions between microglia and oligodendrocytes in MS, as well as on the effects of BBPs on microglia, OPCs or mature oligodendrocytes. It did however leave questions unanswered, or raised new questions to complement new findings.

One topic that could be researched further is that of mGluR modulation as a strategy to prevent microglial induced toxicity to oligodendrocytes. In this study a large amount of data was presented showing that mGluRs do have the ability to modulate microglial activation. For example an mGluR group II agonist and a group III antagonist upregulated iNOS expression in BV-2 cells. The upregulation of iNOS in the presence of group II mGluR agonists was in agreement with evidence for a neurotoxic role for group II mGluR receptors (Taylor et al., 2005). The upregulation of iNOS in the presence of the group III antagonist MAP4 agreed with data for a neuroprotective role for the group III mGluRs (Taylor et al., 2003). Also, in an *in vitro* study, a group III agonist was able to reverse neuronal cell death induced by myelin activated microglia (Pinteaux-Jones et al., 2008). In the chapter investigating LPS induced OPC death, all mGluR agonists and antagonists available were used on microglia in conjunction with LPS to try and ameliorate the toxic effect of LPS activated microglia on OPCs. None of the agonists or antagonists were able to do that. Still, a promising area of research is that of glutamate induced OPC death. It was shown here that OPCs and developing oligodendrocytes but not mature oligodendrocytes were vulnerable to glutamate. It would be interesting to investigate whether glutamate induced OPC death could be prevented by mGluR manipulation. In *in vitro* studies of OPC cells it was shown that group I mGluR activation was able to reverse kainic acid induced OPC toxicity (Kelland & Toms, 2001) or hypoxic-ischemic developing oligodendrocyte injury (Deng et al., 2004). The role of microglial mGluRs in oligodendrocyte toxicity in MS is an interesting field of investigation, since previous observations on neurones point towards an important regulatory role for mGluR receptors in MS lesions. On the other hand mature oligodendrocytes have only been shown to express mGluR3 (Luyt et al., 2006). Thus if



a regulatory role for microglial mGluRs in oligodendrocyte toxicity is revealed, therapeutic avenues directly targeting microglia, OPCs and neurones could be developed.

Another field that deserves further studying is that of differentially activated microglia by BBPs. Although a lot of work was done to elucidate the toxic or protective effects of BBP activated microglia on OPCs or mature oligodendrocytes, and the necessity of cell contact to induce those effects, time constraints did not allow for the study of molecular pathways involved in resulting toxicity or protection. As in the case of LPS activated microglial induced oligodendrocyte death, where TNF- $\alpha$  in conjunction with cell contact was identified as the secreted molecule responsible for microglial mediated cell death, similar experiments should be performed to elucidate BBP mediated oligodendrocyte death. Identification of different protective or toxic cytokines released following differential microglial activation would complement this study. Furthermore, a lot of work could be done to identify the microglial receptors of BBPs. Having excluded BBP signalling via the LPS receptor TLR4, the question of which receptors they act through remains unanswered. Even FG, which has been shown to signal via CD11b (Adams et al., 2007), seems to further bind to other receptors. As suggested in chapter 6, TLR2 receptor, which binds lipoproteins, peptidoglycans and polymeric molecules (Lehnardt, 2009), is a promising candidate for FG and FN ligation.

Finally, in regards to the BBP studies, the finding that FN arrests OPC differentiation should be studied further. Molecular pathways implicated in this mechanism could be studied, and some ideas on which pathways might be involved could be derived from previous studies investigating OPC arrest (Akassoglou et al., 2004; Kotter et al., 2006). The exact developmental stage at which OPCs are preserved when FN is present can be identified by investigating the expression of genes indicative of different OPC maturation stages. There is a wealth of such 'developmental' markers available (Linington et al. 1984; 1988; Nishiyama et al., 1996; Back et al., 2002). This would facilitate the better understanding of the mechanisms behind such differentiation arrest and could help design approaches to induce the cells to differentiate and myelinate.

## 7.4 Conclusion

In conclusion, this thesis has added knowledge to the field of microglial activation in MS and its effects on oligodendrocyte lineage cell viability and maturation. It opened new avenues with the identification that the blood borne proteins FV albumin, fibrinogen and fibrin are able to potently and differentially activate microglia and to induce distinct effects on OPCs or mature oligodendrocytes. It was the first time that a toxic effect to oligodendrocyte lineage cells was attributed to blood-borne proteins and the first time that a blood-borne protein (fibrin) was implicated in myelination failure. Also, new views were added to conflicting issues on mature oligodendrocyte pathology, such as their vulnerability to activated microglia and glutamate. It is understood that basic neuroscience does not directly offer therapy to people suffering from MS, but it is hoped that careful observations of cell biology and its mechanisms will lead to better understanding of pathological events in MS brain tissue and lead to better treatment of this debilitating disease.

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## 9. LIST OF PUBLICATIONS AND CONFERENCES

### Publications

- Davenport CM, Sevastou IG, Hooper C, Pocock JM. Inhibiting p53 pathways in microglia attenuates microglial-evoked neurotoxicity following exposure to Alzheimer peptides. *J Neurochem*. 2010 Jan;112(2):552-63.
- Hooper C, Fry VA, Sevastou IG, Pocock JM. Scavenger receptor control of chromogranin A-induced microglial stress and neurotoxic cascades. *FEBS Lett*. 2009 Nov 3;583(21):3461-6.
- Hooper C, Pinteaux-Jones F, Fry VA, Sevastou IG, Baker D, Heales SJ, Pocock JM. Differential effects of albumin on microglia and macrophages; implications for neurodegeneration following blood-brain barrier damage. *J Neurochem*. 2009 May;109(3):694-705.
- Pinteaux-Jones F, Sevastou IG, Fry VA, Heales S, Baker D, Pocock JM. Myelin-induced microglial neurotoxicity can be controlled by microglial metabotropic glutamate receptors. *J Neurochem*. 2008 Jul;106(1):442-54.

### Conferences

- 9<sup>th</sup> European Meeting on Glial Cells in Health and Disease, 8-12 September 2009, Paris, France  
Poster presentation: "Modulation of microglial induced oligodendrocyte toxicity in multiple sclerosis".
- 22<sup>nd</sup> Annual Meeting of the Hellenic Society For Neuroscience 16-19 October 2008, Athens, Greece
- Multiple Sclerosis: From Pathogenesis to Therapy, June 6, 2008 Paris, France
- Neuroscience 2007, Society for Neuroscience (SfN) annual meeting, 3-7 November 2007, San Diego, California, USA  
Poster presentation: "Microglial ROCK and Rho in multiple sclerosis".
- 8<sup>th</sup> Annual Queen Square Symposium 2007, Queen Square, London, UK  
Poster presentation: "Can manipulation of microglial mGlu receptors affect oligodendrocyte survival in multiple sclerosis?".